GENETIC DIVERSITY AND PHYLOGEOGRAPHY OF THE BLACKLEGGED TICK, *IXODES SCAPULARIS*, AND AN ASSOCIATED BACTERIUM, *ANAPLASMA PHAGOCYTOPHILUM*

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Abstract

Climate change is altering the geographical distributions of arthropod vectors (e.g., mosquitoes and ticks) and their associated microorganisms, some of which are pathogenic to humans and/or animals. It is important to determine the origin(s) of vectors in newly established populations, particularly if there are geographical differences in the species and strains of pathogens they carry. The blacklegged tick, *Ixodes scapularis*, is a key vector of pathogens in the U.S.A., and its distributional range continues to expand within Canada. The aim of my Ph.D. research was to genetically characterize *I. scapularis* individuals and an associated bacterium, *Anaplasma phagocytophilum* (i.e., the causative agent of human granulocytic anaplasmosis (HGA)), and to infer the geographical origins of the different populations of this tick species in Canada.

Several genetic markers were used to characterize blacklegged ticks collected from different localities in Canada and the U.S.A. and to determine the phylogeographical relationships among different sequence variants (i.e., haplotypes). A major discovery was that the genetic variability in *I. scapularis* was much greater than previously reported. There were also major differences in the occurrence of many haplotypes among geographical regions, suggesting different geographical origins for some tick populations in Canada. These differences may be associated with the different major migratory flyways that passerines, which transport large numbers of immature *I. scapularis* from the U.S.A. into Canada, use each spring. A shallow phylogeographical pattern was observed for *I. scapularis*, which was consistent with the life history of a generalist tick species that is dispersed over large geographical areas by migratory birds. The phylogeographical data also suggested that *I. scapularis* populations in Manitoba likely originated from those in Minnesota, whereas tick populations in southeastern Ontario probably originated from those in neighbouring states of the Northeast, U.S.A. Thus, ticks in the Midwest, U.S.A. (e.g., Minnesota) may be transporting different species and strains of pathogens into Canada than those in the Northeast (e.g., Rhode Island).

Molecular assays targeting the 16S rRNA gene of *A. phagocytophilum* from infected ticks detected both the strain associated with HGA (i.e., Ap-ha), and a strain not associated with human infection (i.e., Ap-variant 1). PCR-based assays were developed to discriminate between the two strains, which enhances the ability of public health officials to assess the risk of exposure of Canadians to HGA. The proportion of infected ticks that contained the Ap-ha strain was

higher in Manitoba than in more eastern provinces of Canada, suggesting that the risk of human exposure to the Ap-ha strain differs among geographical areas. Phylogenetic and phylogeographical analyses of DNA sequences of the ankyrin (*ankA*) gene of *A. phagocytophilum* revealed deep genetic structure, but common lineages were sympatric over a large geographical area. Thus, the phylogeographical patterns observed for *A. phagocytophilum* were incongruous with that for *I. scapularis*, suggesting that genetic variants of *I. scapularis* cannot be used to infer or predict where particular strains of *A. phagocytophilum* are likely to occur in Canada. However, the most common *ankA* strains of *A. phagocytophilum* varied among geographical regions, possibly in accordance with the different flyways used by the migratory passerines that are transporting blacklegged ticks, supporting the hypothesis of different origins for some tick populations in Canada.

In conclusion, the work conducted herein makes a valuable contribution to our understanding of the population genetics of *I. scapularis*, and the phylogeographical relationships among different sequence variants of *I. scapularis* and *A. phagocytophilum* in Canada. The findings of this thesis may also have implications for studies on other arthropod vectors and their associated pathogens whose distributional ranges may also be changing in response to climate change.

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List of Abbreviations

AB	Alberta
AMOVA	Analysis of molecular variance
ankA	Ankyrin A
CGE	Canine granulocytic ehrlichiosis
CI	Confidence interval
cox1	Cytochrome c oxidase subunit 1
cox2	Cytochrome c oxidase subunit 2
cox3	Cytochrome c oxidase subunit 3
CR	Camp Ripley
CSP	St. Croix State Park
C_T	Cycle threshold
CT	Connecticut
cytb	Cytochrome b
DNA	Deoxyribonucleic acid
FAM	6-carboxy-fluorescein
F_S	Fu's F_S statistic
F_{ST}	Fixation index
g	Genomic
gltA	Citrate synthase
h	Haplotype diversity
HGA	Human granulocytic anaplasmosis
HGE	Human granulocytic ehrlichiosis
HISK	Hazard Island, South Kingstown
HT	Haplotype
ISP	Itasca State Park
LPPP	Long Point Provincial Park
LSU	Large subunit
MA	Massachusetts
MB	Manitoba

MD	Maryland
MGB	Minor groove binder
MN	Minnesota
MP	Maximum parsimony
mt	Mitochondrial
n	Nuclear
n	Sample size
π	Nucleotide diversity
NB	New Brunswick
NCBI	National Center for Biotechnology Information
NFQ	Non-fluorescent quencher
NJ	Neighbour joining
NL	Newfoundland
NML	National Microbiology Laboratory
nPCR	Nested polymerase chain reaction
NRC	National Research Council
NS	Nova Scotia
NY	New York
ON	Ontario
ospC	Outer surface protein C
PA	Pennsylvania
PCR	Polymerase chain reaction
PE	Prince Edward Island
POW	Powassan virus
PPNP	Point Pelee National Park
PVPP	Pembina Valley Provincial Park
QC	Quebec
r	Ribosomal
RFLP	Restriction fragment length polymorphism
RI	Rhode Island
RMSF	Rocky mountain spotted fever

SK	Saskatchewan
SNP	Single nucleotide polymorphism
SSCP	Single-strand conformation polymorphism
SSU	Small subunit
ST	Stanley Trail
t	Transfer
TPSK	Trustom Pond, South Kingstown
$tRNA^{Val}$	Transfer ribonucleic acid (valine)
TROSPA	Tick receptor for the *Borrelia burgdorferi* outer surface protein A
U of S	University of Saskatchewan
WI	Wisconsin

CHAPTER 1
INTRODUCTION

1.1. Arthropods and arthropod-borne pathogens

Arthropods, such as mosquitoes, flies, triatomine bugs, fleas, and ticks, are the most important vectors of human and animal pathogenic agents (i.e., bacteria, viruses, and protozoa) in the world (Gratz 1999, Parola and Raoult 2001, Stratton et al. 2008, Zhang et al. 2008, Ooi and Gubler 2009, Colwell et al. 2011, Klotz et al. 2014, Montgomery et al. 2014). For example, mosquitoes of the genus *Anopheles* are the vectors of four different protozoa (i.e., *Plasmodium* spp.) that are the causative agents of malaria, a disease with an incidence estimated at 300-500 million new clinical cases per annum in over 92 countries in the tropics and subtropics (Gratz 1999, Martens et al. 1999, Zhang et al. 2008). In more temperate areas, including North America, ticks are the most important vectors of pathogenic agents to humans and domestic animals (Parola and Raoult 2001). Tick-borne diseases in North America include Lyme disease, anaplasmosis, ehrlichiosis, and Rocky Mountain spotted fever (RMSF) (Oliver 1989, Parola and Raoult 2001, Piesman and Eisen 2008).

The distribution and abundance of many species of arthropods are limited by abiotic factors, because the development times and/or survival rates of their different life cycle stages are dependent upon the temperatures and relative humidities of the microenvironments in which they live (Lindsay et al. 1995, Bertrand and Wilson 1996, Lindsay et al. 1998, 1999a, Gubler et al. 2001, Ogden et al. 2005, Koenraadt et al. 2006, Torremorell and Leman 2010, Kingsolver et al. 2011, Myaing 2011, Lyons et al. 2013). Thus, regional changes in climatic conditions can lead to changes in the distribution and abundance of arthropods and the prevalences of arthropod-borne diseases (Githeko et al. 2000, Yoganathan and Rom 2001, Brownstein et al. 2005, Harrus and Baneth 2005, Ogden et al. 2005, Chaves and Pascual 2006, Ogden et al. 2006b, 2008a, 2008d, Zhang et al. 2008, Ogden et al. 2009, Ostfeld 2009, Hongoh et al. 2012, Leighton et al. 2012, Moore et al. 2012, Gallana et al. 2013, Medlock et al. 2013, Ogden et al. 2013b). There is substantial evidence that climate change has occurred worldwide (Yoganathan and Rom 2001, Folland et al. 2002). Global surface temperatures have linearly increased by 0.6ºC between 1861 and 2000 (Folland et al. 2002). Also, land surface precipitation has increased in the mid- and high-latitudes of the Northern Hemisphere by 0.5-1% per decade and in tropical areas by

2.4% per century, but has decreased at a rate of 0.3% per decade in subtropical regions (Folland et al. 2002). Such large-scale changes have resulted in the emergence and resurgence of arthropod-borne diseases, particularly those transmitted by mosquitoes (Gratz 1999, Githeko et al. 2000, Kovats et al. 2001, Yoganathan and Rom 2001, Harrus and Baneth 2005, Cohen et al. 2012, Hongoh et al. 2012, Gallana et al. 2013) and ixodid ticks (Gratz 1999, Githeko et al. 2000, Lindgren et al. 2000, Kovats et al. 2001, Yoganathan and Rom 2001, Harrus and Baneth 2005, Ogden et al. 2006b, 2008a, 2008c, 2008d, 2009, Gilbert 2010, Medlock et al. 2013, Ogden et al. 2013a, 2013b).

Understanding the ecology of vector-borne diseases requires detailed knowledge of the biology, ecology, population genetics, and phylogeography of both the vector and the pathogens it transmits to its vertebrate hosts (Kurtenbach et al. 2006). The study of population genetics provides information about the biological, demographic, and historical processes affecting populations, as sequence variation within a species is shaped by natural selection, as well as chance (Chakravarti 1999). For example, examination of the population genetics of the vector can indicate if there are physical or ecological barriers to gene flow among populations, which has important implications with respect to the transmission of vector-borne pathogens (Norris et al. 1996, McCoy et al. 2003, Noureddine et al. 2011, Margos et al. 2012, Rogic et al. 2013, Dinnis et al. 2014). Both mitochondrial (mt) and nuclear (n) DNA have been used to study the population genetics of a wide range of arthropods (Lanzaro et al. 1995, France and Kocher 1996, Lehmann et al. 1996, Norris et al. 1996, McCoy et al. 2001, Birungi and Munstermann 2002, Hodgkinson et al. 2002, Qiu et al. 2002, Marquez and Krafsur 2003, McCoy et al. 2003, Bailly et al. 2004, Rosenthal and Spielman 2004, Austin et al. 2005, Timmermans et al. 2005, Luchetti et al. 2006, Rugman-Jones et al. 2007, Mahani et al. 2009, Krakowetz et al. 2010, Bataille et al. 2011, Krakowetz et al. 2011, Zahedani et al. 2011, Ferrari et al. 2013, Pfeiler et al. 2013). A variety of PCR-based methods have also been employed to examine genetic variability within select arthropod vectors (Ballingercrabtree et al. 1992, Norris et al. 1996, Cushing 1998, Birungi and Munstermann 2002, Hodgkinson et al. 2002, Qiu et al. 2002, Marquez and Krafsur 2003, Timmermans et al. 2005, Patterson et al. 2009, Krakowetz et al. 2010, Lambeets et al. 2010, Krakowetz et al. 2011, Ferrari et al. 2013). For example, PCR-based single-strand conformation polymorphism (SSCP) is a simple and cost-effective method to examine genetic variation among individuals within a population (Gasser et al. 2006). This mutation scanning technique is based

on the principle that single-stranded DNA molecules migrate through a non-denaturing gel based on their size and sequence (i.e., their secondary or tertiary structure due to base pairing between nucleotides within individual strands) when subjected to electrophoresis (Gasser et al. 2006). SSCP can differentially display DNA fragments of approximately 100-500 bp that differ by one or more nucleotides (Gasser et al. 2006). This approach, in combination with DNA sequencing, has been used to pre-screen for genetic variation in a wide variety of arthropods (Norris et al. 1996, Birungi and Munstermann 2002, Hodgkinson et al. 2002, Qiu et al. 2002, Marquez and Krafsur 2003, Patterson et al. 2009, Krakowetz et al. 2010, 2011, Ferrari et al. 2013) and among strains of arthropod-borne pathogenic bacteria (Qiu et al. 1997, Norris et al. 1999, Qiu et al. 2002, Derdakova et al. 2003, Lagal et al. 2003, Swanson and Norris 2008, Humphrey et al. 2010, Dergousoff and Chilton 2011). Determining the extent and spatial distribution of the genetic variation within and among populations of vectors in a geographical and historical context can provide insights into the colonization events that led to their current geographical distributions (Bohonak 1999, Chakravarti 1999). Such information may also provide insights into the possible changes in the distributional range of arthropod vectors and their pathogens in the future.

Phylogeography is the study of the evolutionary relationships of individuals within populations of a species in a biogeographical context (Avise 2000). This interdisciplinary field requires information from population genetics, phylogenetics, paleontology, geology, ethology, demography, and historical geography (Avise 2000). In phylogeographical studies, the choice of genetic marker is important. The ideal marker must be sufficiently variable to detect phylogeographical patterns, but not so variable as to make these patterns difficult to discern. Although nDNA, including microsatellite loci, has been used in phylogeographical studies (Avise 2000, Araki et al. 2008, Hu et al. 2008, Hickerson et al. 2010, Noureddine et al. 2011, Song et al. 2011, Beati et al. 2012, Kindler et al. 2012, Kovalev and Mukhacheva 2012, Paupy et al. 2012, Beati et al. 2013, Brown et al. 2013, Cangi et al. 2013, Jeratthitikul et al. 2013, Matallanas et al. 2013, Nistelberger et al. 2014, Schrimpf et al. 2014), mtDNA is often the primary target (Marquez et al. 2007, Casati et al. 2008, Hafner et al. 2008, Rajabi-Maham et al. 2008, Gaubert et al. 2009, Harris and Perera 2009, Minamiya et al. 2009, Trout et al. 2009, Humphrey et al. 2010, Noureddine et al. 2011, Song et al. 2011, Beati et al. 2012, Kindler et al. 2012, Kovalev and Mukhacheva 2012, Paupy et al. 2012, Beati et al. 2013, Cangi et al. 2013, Jeratthitikul et al. 2013, Mechai et al. 2013, Nistelberger et al. 2014, Schrimpf et al. 2014),

because it evolves faster than single-copy nDNA (Avise 2000). Hence, mtDNA is often more variable among individuals within populations than nDNA (Beati et al. 2012, Kovalev and Mukhacheva 2012, Beati et al. 2013, Cangi et al. 2013). For example, in a recent phylogeographical study by Kovalev and Mukhacheva (2012), the extent of the genetic variability within a portion of the mt 12S ribosomal (r) RNA gene (i.e., four sequence types; $n = 76$) was shown to be greater than that within a fragment of the D3 domain and flanking core regions of the 28S rRNA gene (i.e., a single sequence type; $n = 25$) of *Ixodes persulcatus* collected from several localities in Russia. The authors proposed that the homogeneity of *I. persulcatus* across Russia was more likely due to the impact of global climate changes than the recent origin of the species (Kovalev and Mukhacheva 2012); however, this hypothesis remains to be tested for *I. persulcatus*, as well as for several other species of tick (e.g., *Ixodes ricinus*) whose distributions are also being affected by climatic changes (Medlock et al. 2013).

1.2. Ixodid ticks

There are over 690 species of tick that belong to the family Ixodidae (Oliver 1989, Parola and Raoult 2001). Ixodid or "hard" ticks are separated into two major groups: the Prostriata, which consists of species within the genus *Ixodes*, and the Metastriata, which is comprised of several genera: *Amblyomma*, *Aponomma*, *Haemaphysalis*, *Hyalomma*, *Cosmiomma*, *Dermacentor*, *Rhipicentor*, *Anomalohimalaya*, *Nosomma*, *Rhipicephalus*, *Boophilus*, and *Margaropus* (Oliver 1989, Parola and Raoult 2001). These two major groups of ticks can be distinguished from one another on the basis of the presence (Prostriata) or absence (Metastriata) of a pre-anal groove (Klompen et al. 1997). There are several other morphological and biological differences between the Prostriata and Metastriata, such as differences in the distributional patterns of sensory organs (termed lyrifissures) (Kahn 1964, Klompen et al. 1997). The Prostriata is comprised of approximately 240 species, one of which is *Ixodes scapularis* (Oliver 1989, Black and Piesman 1994).

Although there is variability among members of the family, ixodid ticks are not particularly resistant to desiccation and starvation; thus, most are short-lived (i.e., the life spans of ixodid ticks can reach four years, but, generally, do not exceed three) (Lindsay et al. 1998, Parola and Raoult 2001). Ixodid ticks feed for prolonged periods of time on their hosts, as compared to many other arthropod vectors (e.g., mosquitoes), and ingest large volumes of blood

compared to their own body mass (i.e., ~100 times) (Parola and Raoult 2001, Latif et al. 2012). They also tend to inhabit above-ground environments (e.g., scrub, moorlands, pastures, meadows, savannah, forest, and brush), with the exception of some nidicolous species within the genus *Ixodes* (especially the immatures; that is, larvae and nymphs), which live in or near the shelters used by their nest-, cave-, crevice-, and/or burrow-dwelling hosts (Oliver 1989, Parola and Raoult 2001, Anderson 2002, Sonenshine and Roe 2014b). However, hard ticks spend the majority of their time in the off-host environment and very little time, relatively, on their host animals (i.e., 3-5 weeks) (Anderson 2002).

The life cycle of ixodid ticks consists of four stages: egg, larva, nymph, and adult (male and female) (Oliver 1989), each of which (except for the egg stage) requires a host for nourishment. The general life cycle of a three-host ixodid tick is as follows. Mated female ticks that have fed (i.e., engorged) to repletion on a host will detach from the host, lay their eggs on the ground in large batches, and then die (Oliver 1989, Anderson 2002). Once the eggs have hatched, the resulting larvae seek out a suitable host to which they attach and feed on for a period of a few days to several weeks (Bull et al. 1989, Oliver 1989, Chilton and Bull 1991, Anderson 2002). Significantly longer feeding times by larvae have been observed for species feeding on reptiles, as compared to those feeding on mammals and birds (Bull et al. 1989, Oliver 1989, Chilton and Bull 1991, Anderson 2002). Once engorged to repletion, larvae detach from the host and molt through to the nymphal stage (Oliver 1989, Anderson 2002). Then nymphs find another host on which to feed (i.e., 4-8 days or 3-6 weeks, depending on type of host parasitized) (Bull et al. 1989, Oliver 1989, Chilton and Bull 1991, Anderson 2002). After engorging to repletion, nymphs detach from the host and molt to the adult stage (Bull et al. 1989, Oliver 1989, Chilton and Bull 1991, Anderson 2002). Adult ticks then seek out and attach to a third host (Oliver 1989). Males ingest significantly smaller blood meals than mated female ticks (Oliver 1989). For many species, mating occurs on the host, once females have spent a few days feeding on a host (Oliver 1989). However, some species have adapted their feeding behaviours so that they require fewer hosts to complete their life cycle (Oliver 1989). The life cycle of a two-host tick is similar to that of a three-host tick except that nymphs and larvae feed on the same individual host, because the engorged larvae molt on the host (Oliver 1989). For one-host ticks, the same individual host is used by the larval, nymphal, and adult stages, because the engorged larvae and

engorged nymphs remain in contact with the host during ecdysis (i.e., molting to the next life cycle stage) (Oliver 1989).

1.3. Ticks and tick-borne pathogens in North America

Many species of tick transmit bacterial, viral, and protozoan pathogens to the hosts they parasitize, which include humans, domestic animals, and wildlife (Estrada-Peña and Jongejan 1999, Parola and Raoult 2001, Piesman and Eisen 2008). In North America, there are more than 30 species of ixodid tick representing at least five genera (Bishopp and Trembley 1945). These include several pest species (e.g., the brown winter tick, *Dermacentor nigrolineatus*; the tropical horse tick, *Dermacentor nitens*; the iguana tick, *Amblyomma dissimile*; and *Ixodes auritulus*), which are of little or no economic importance, and some species (e.g., the rabbit tick, *Haemaphysalis leporis-palustris*; and the rotund tick, *Ixodes kingi*) that are capable of carrying disease-causing agents of humans (e.g., the causative agents of tularemia and RMSF) (Bishopp and Trembley 1945). These also include several tick species that feed on humans, as well as vector pathogens of public health importance (e.g., the lone star tick, *Amblyomma americanum*, the wood tick, *Dermacentor andersoni*, and the blacklegged tick, *I. scapularis*) (Bishopp and Trembley 1945). Table 1.1 provides a summary of the species of ixodid ticks in North America that are of public health significance because of the pathogens that they carry (Hearle 1938, McLean and Donohue 1959, Spielman 1976, Spielman et al. 1979, Burgdorfer et al. 1982, Johnson et al. 1984, Barker et al. 1993, Bakken et al. 1994, Chen et al. 1994, Costero and Grayson 1996, Keirans et al. 1996, Hodzic et al. 1998, Estrada-Peña and Jongejan 1999, Burkot et al. 2001, James et al. 2001, Parola and Raoult 2001, Gary et al. 2006, Piesman and Eisen 2008, Jordan et al. 2009, Scott et al. 2010, Dergousoff et al. 2013). These ticks belong to four genera (i.e., *Ixodes*, *Amblyomma*, *Dermacentor*, and *Rhipicephalus*) and are the principal vectors of pathogenic agents to humans in North America.

Pathogenic agents are transmitted by ticks in several ways (Fine 1975, Gordh and Headrick 2001, Parola and Raoult 2001, Baldridge et al. 2009, Davis and Bent 2011, Heylen and Matthysen 2011, Nefedova et al. 2012). Some are transmitted transovarially; that is, they are passed from an adult female to her eggs via her infected ovaries (Ergonul and Whitehouse 2007). This type of transmission, as well as transovum transmission (i.e., passage from an adult female directly to her eggs), is a mode of vertical transmission (Fine 1975, Tsao 2009). Transovarial

Table 1.1. Summary of ixodid species occurring in Canada and/or the U.S.A. that feed on humans and vector pathogens of public health importance.

Ixodid species	Distribution	Pathogen(s)/Causative agent(s)	Disease(s)	Reference(s)
Amblyomma spp.				
A. americanum	Canada (central Alberta), central & eastern U.S.A., Central & South America	*Francisella tularensis* *Rickettsia rickettsii* *Ehrlichia chaffeensis* *Borrelia lonestari* *Coxiella burnetii*	Tularemia Rocky Mountain spotted fever Human monocytic ehrlichiosis Southern tick-associated rash illness Q-fever	Estrada-Peña and Jongejan 1999, Burkot et al. 2001, James et al. 2001, Parola and Raoult 2001, Piesman and Eisen 2008, Jordan et al. 2009, Scott et al. 2010
A. cajennense	southern Texas, Mexico, Central & South America	*R. rickettsii*	Rocky Mountain spotted fever	Estrada-Peña and Jongejan 1999, Parola and Raoult 2001, Piesman and Eisen 2008
A. maculatum	southern Canada, U.S.A., South America (Argentina)	*R. rickettsii*	Rocky Mountain spotted fever	Estrada-Peña and Jongejan 1999, Scott et al. 2010
Dermacentor spp.				
D. andersoni	western Canada (including Alberta & Saskatchewan), western U.S.A.	*R. rickettsii* *Coltivirus* *F. tularensis* *C. burnetii*	Rocky Mountain spotted fever Colorado tick fever Tularemia Q-fever	Estrada-Peña and Jongejan 1999, Parola and Raoult 2001, Piesman and Eisen 2008, Dergousoff et al. 2013
D. variabilis	central & eastern Canada (also Saskatchewan & Manitoba), U.S.A. (except Rocky Mountains), Mexico	*R. rickettsii* *F. tularensis*	Rocky Mountain spotted fever Tularemia	Estrada-Peña and Jongejan 1999, Parola and Raoult 2001, Piesman and Eisen 2008, Dergousoff et al. 2013
D. occidentalis	western U.S.A., Mexico	*R. rickettsii* *F. tularensis* *Coltivirus*	Rocky Mountain spotted fever Tularemia Colorado tick fever	Estrada-Peña and Jongejan 1999

Table 1.1. Continued.

Ixodid species	Distribution	Pathogen(s)/Causative agent(s)	Disease(s)	Reference(s)
Ixodes spp.				
I. scapularis (formerly *I. dammini*)	central & eastern Canada (also Alberta, Saskatchewan & Manitoba), central & eastern U.S.A.	*Borrelia burgdorferi* sensu stricto *Anaplasma phagocytophilum* *Babesia microti* Powassan virus	Lyme disease Human granulocytic anaplasmosis Human babesiosis Powassan encephalitis	McLean and Donohue 1959, Spielman 1976, Spielman et al. 1979, Burgdorfer et al. 1982, Johnson et al. 1984, Bakken et al. 1994, Chen et al. 1994, Costero and Grayson 1996, Keirans et al. 1996, Hodzic et al. 1998, Estrada-Peña and Jongejan 1999
I. pacificus	western Canada, western U.S.A. (also Arizona), Mexico	*B. burgdorferi* s.s. *A. phagocytophilum*	Lyme disease Human granulocytic anaplasmosis	Dennis et al. 1998, Estrada-Peña and Jongejan 1999, Parola and Raoult 2001, Piesman and Eisen 2008
I. cookei	eastern Canada, eastern & Midwestern U.S.A.	Powassan virus	Powassan encephalitis	Barker et al. 1993, Estrada-Peña and Jongejan 1999
I. dentatus	Canada (British Columbia), Eastern U.S.A.	*B. burgdorferi* s.s. *F. tularensis* *C. burnetii*	Lyme disease Tularemia Q-fever	Hearle 1938, Estrada-Peña and Jongejan 1999, Parola and Raoult 2001, Piesman and Eisen 2008
Rhipicephalus spp.				
R. sanguineus	Canada, U.S.A. (also occurs in several parts of the Old World)	*R. rickettsii*	Rocky Mountain spotted fever	Estrada-Peña and Jongejan 1999, Parola and Raoult 2001, Wood and Artsob 2011

transmission is exhibited by several pathogens transmitted by North American ticks of medical and veterinary significance (Table 1.1), such as *Francisella tularensis* (in *A. americanum*, *D. andersoni*, *Dermacentor variabilis*, and *Ixodes dentatus*), *Coxiella burnetii* (in *A. americanum*, *D. andersoni*, and *I. dentatus*), and *Rickettsia rickettsii* (in *Amblyomma cajennense*, *D. andersoni*, and *D. variabilis*) (Parola and Raoult 2001).

Disease-causing agents in ticks can also be retained or passed from one active life cycle stage of a tick to another (e.g., from a larva to a nymph or from a nymph to an adult) (Gordh and Headrick 2001, Ergonul and Whitehouse 2007). This process of transmitting pathogens between different feeding stages is termed transstadial transmission (Gordh and Headrick 2001, Ergonul and Whitehouse 2007). Transstadial transmission is exhibited by several pathogens vectored by North American ticks of public health significance (Table 1.1), such as *F. tularensis* (in *A. americanum*, *D. andersoni*, *D. variabilis*, and *I. dentatus*), *R. rickettsii* (in *A. cajennense*, *D. andersoni*, and *D. variabilis*), *Borrelia burgdorferi* (in *Ixodes pacificus* and *I. dentatus*), and *Anaplasma phagocytophilum* (in *I. pacificus*) (Parola and Raoult 2001).

Another form of pathogen transmission in ticks is horizontal transmission, which occurs when pathogens are transferred between ticks and their vertebrate hosts (Tsao 2009). Horizontal transmission via systemic infection is the most commonly recognized route of transmission between an infected vertebrate host and uninfected ticks (Davis and Bent 2011). However, transmission by co-feeding also occurs (Randolph et al. 1996, Tsao 2009, Davis and Bent 2011). Co-feeding transmission happens when susceptible ticks feed simultaneously with infected ticks on the same uninfected host animal and become infected (Randolph et al. 1996, Tsao 2009). This form of pathogen transmission occurs even when there is physical separation (>1 cm) between the uninfected and infected ticks (Randolph et al. 1996). Co-feeding transmission also describes the process whereby susceptible ticks acquire infections from infected ticks by feeding from the same site on the host animal at which infected ticks had recently fed (Randolph et al. 1996). Thus, simultaneous feeding does not necessarily have to occur for co-feeding transmission to take place (Randolph et al. 1996). Such "extended co-feeding transmission" is characteristic of *B. burgdorferi*, because *Borrelia* are unable to spread quickly within the vertebrate host due to their limited mobility (Randolph et al. 1996). In contrast, "co-feeding transmission" is typical of tick-borne viruses, as viruses infect cells of the host (e.g., Langerhans cells) and disseminate rapidly within the lymphatic system of the host (Randolph et al. 1996).

Tick saliva is rich in bioactive components that can interfere with coagulation, platelet aggregation, and vasodilation in the vertebrate host (Randolph et al. 1996). Such components may also influence the transmission of pathogens in ways that vary by pathogen, vector, and/or vertebrate host (Randolph et al. 1996). For example, proteins produced in the salivary glands of the tick that become secreted in the saliva can promote the transmission of viruses (Randolph et al. 1996, Nuttall and Labuda 2004, Machackova et al. 2006, Horka et al. 2009). The term for this type of transmission is saliva-activated transmission (Randolph et al. 1996, Nuttall and Labuda 2004, Machackova et al. 2006, Horka et al. 2009). Direct evidence of saliva-activated transmission has been reported for *B. burgdorferi* by *I. scapularis* (Zeidner et al. 2002).

1.4. The blacklegged tick, *Ixodes scapularis*

1.4.1. Morphological characteristics

Ixodes scapularis Say 1821, commonly referred to as the blacklegged tick (Keirans et al. 1996) or deer tick (Wilson et al. 1984, Wilson et al. 1985), is a member of the *Ixodes ricinus* complex. This species complex contains at least 13 other species of *Ixodes* that are distributed throughout the world: *I. ricinus*, *I. pacificus*, *Ixodes pavlovskyi*, *I. persulcatus*, *Ixodes nipponensis*, *Ixodes gibbosus*, *Ixodes jellisoni*, *Ixodes pararicinus*, *Ixodes affinis*, *Ixodes muris*, *Ixodes minor*, *Ixodes granulatus* and *Ixodes nuttallianus* (Xu et al. 2003). The primary vectors of the spirochetes that cause Lyme disease (i.e., Lyme borreliosis) belong to this complex (Xu et al. 2003). In North America, *I. scapularis* and *I. pacificus* are the main vectors of the Lyme disease spirochetes (Xu et al. 2003).

The morphological description of *I. scapularis* is based on a female specimen collected from an unspecified locality in the U.S.A. (see Keirans et al. 1996). Subsequently, a new species, *Ixodes dammini* Spielman, Clifford, Piesman, and Corwin, 1979 was described (Spielman et al. 1979). Specimens of *I. dammini* differed from *I. scapularis* based on several morphological characters including shorter spiracular plates (i.e., a structure situated on the ventral idiosomal surface of adult and nymphal ticks containing the spiracles; the openings to the respiratory system) on males, a shorter and broader internal coxal spur I (i.e., a projection extending posteriorly from a coxa; the first, basal segment of a leg) on males and females, but primarily based on the larger auriculae (i.e., paired extensions on the ventral surface of the basis capituli; a

ring of cuticle to which the hypostome, chelicerae, and palps are attached) of nymphs (Spielman et al. 1979, Keirans et al. 1996, Sonenshine and Roe 2014a). However, it was later determined that *I. scapularis* and *I. dammini* represented a single species based on hybridization and assortive mating experiments, and comparative analyses of the morphometrics, chromosomes, and isozyme analyses of individuals of the two taxa (Oliver et al. 1993b). As a consequence, *I. dammini* was reduced to a junior subjective synonym of *I. scapularis* (Oliver et al. 1993b).

Several morphological keys have been developed for the identification of the larvae, nymphs, and adults of *I. scapularis* (Durden and Keirans 1996, Kleinjan and Lane 2008, Guzmán-Cornejo and Robbins 2010). Morphologically, females of *I. scapularis* can be distinguished from females of other North American species of *Ixodes* by the presence of small, but distinct cornua (i.e., paired posterolateral protrusions located on the dorsum of the capitulum), as well as a nearly circular scutum with larger punctations near its periphery (Guzmán-Cornejo and Robbins 2010, Sonenshine and Roe 2014a). Punctations are the pits or depressions that are present on the surface of the hardened cuticle (Sonenshine and Roe 2014a). Males of *I. scapularis* can be distinguished from males of other North American species of *Ixodes* by the presence of an elongate spiracular plate and a median plate with large punctations (Guzmán-Cornejo and Robbins 2010). The spiracular plate is a structure situated on the ventral idiosomal surface of adult and nymphal ticks (Sonenshine and Roe 2014a).

1.4.2. Distribution

The distributional ranges of ticks are based on the locations of "established" (i.e., reproducing) populations; however, different definitions have been used to define an established population of *I. scapularis*. Dennis et al. (1998) considered blacklegged ticks to be established in a county of the U.S.A. if at least six ticks (irrespective of life cycle stage) were collected, or if individuals of at least two of the three active life cycle stages (i.e., larvae, nymphs, and adults) were collected. In contrast, an established population of *I. scapularis* in Canada has been defined as one that occurs in an area where all three active stages are found in a contiguous sampling area (i.e., locality) on resident animals or in the environment for a minimum of two consecutive years (Laboratory Center for Disease Control et al. 1991).

Established populations of *I. scapularis* in the U.S.A. have been detected in 396 counties within 32 states (Dennis et al. 1998). These populations are concentrated along the northeastern

Atlantic Coast from Maine to Maryland and from North Carolina to Georgia, and along the Gulf Coast of the United States from Florida to Texas (Dennis et al. 1998). Clusters of established populations also occur in Arkansas and Missouri (in the Southwest Midwest) and in Minnesota, Wisconsin, Michigan, and Indiana (in the Upper Midwest), the latter of which remain geographically isolated from those along the coasts and in the Southwest Midwest (Dennis et al. 1998, Diuk-Wasser et al. 2006).

In Canada, the first established population of *I. scapularis* was detected in Long Point Provincial Park, Ontario in the early 1970s (Watson and Anderson 1976). Additional established populations in Point Pelee National Park and Rondeau Provincial Park, Ontario were not detected until the late 1990s (Barker and Lindsay 2000). Since then, the distribution of this tick species in Canada has continued to expand, with geographically isolated populations establishing in Manitoba, Ontario, Quebec, New Brunswick, and Nova Scotia (Ogden et al. 2005, 2008b, 2008c, 2009, Bouchard et al. 2011, Koffi et al. 2012). This rapid expansion has been attributed to climatic conditions becoming more favourable for the survival and reproduction of this tick (Ogden et al. 2006b, 2008a) and to the continual transportation of larval and nymphal *I. scapularis* from the U.S.A. by migratory passerines (Klich et al. 1996, Scott et al. 2001, Morshed et al. 2005, Ogden et al. 2008c, Scott et al. 2010, 2012). The transportation of immature ticks into Canada via migratory birds has resulted in abundant reports of "adventitious" *I. scapularis* in every Canadian province east of Alberta, including Saskatchewan (Ogden et al. 2006c, 2010, Anstead and Chilton 2011, Bouchard et al. 2011, Krakowetz et al. 2011, Koffi et al. 2012, Ogden et al. 2013a). Adventitious ticks are defined as those that occur sporadically, both temporally and spatially (Laboratory Center for Disease Control et al. 1991). Although the distribution of *I. scapularis* has expanded rapidly in recent years and is expected to continue expanding at a rate of 46 km per year for the next decade, it has been predicted that climatic changes associated with global warming will accelerate the rate of spread (Leighton et al. 2012).

1.4.3. Life cycle, seasonal activity patterns, and hosts

In the Midwest and Northeast, U.S.A., the results of field-based studies suggest that the life cycle of *I. scapularis* takes 2-4 years to complete, depending on host availability (Anderson and Magnarelli 1980, Yuval and Spielman 1990, Platt et al. 1992). For the most part, the life cycle of *I. scapularis* in these two geographical regions is similar (Yuval and Spielman 1990,

Platt et al. 1992); however, there are differences in seasonal activity of the larval life cycle stage between regions (Platt et al. 1992, Gatewood et al. 2009). In the Upper Midwest, larval activity takes place over a broader period of time (late April through October) and peaks earlier (July) than in the Northeast (August) (Platt et al. 1992, Gatewood et al. 2009). Adults, which are active during the fall (peak densities = mid-October) and spring (peak densities = mid-May), must obtain a blood meal from a suitable host prior to the following summer, otherwise they will perish (Yuval and Spielman 1990, Siegel et al. 1991, Platt et al. 1992). Blood-fed females (April) lay their eggs in early summer (early June in the Midwest or mid-to-late May in the Northeast), regardless of whether they obtained their meals during the previous year's fall or the present year's spring (Yuval and Spielman 1990, Platt et al. 1992, Daniels et al. 1996). Eggs of female *I. scapularis* hatch synchronously (July), resulting in a peak of larval activity in late summer (July in the Midwest or August in the Northeast) (Yuval and Spielman 1990, Platt et al. 1992, Daniels et al. 1996, Gatewood et al. 2009). If larvae obtain a blood-meal by fall (September), they will molt and overwinter as nymphs (Yuval and Spielman 1990). If they cannot feed by fall (September), then they will overwinter unengorged and, subsequently, obtain blood-meals and molt to nymphs in the spring (peak nymphal densities = June, but season = May through August) (Yuval and Spielman 1990, Siegel et al. 1991, Platt et al. 1992, Gatewood et al. 2009). Larval cohorts (i.e., a group of ticks that began a given life cycle stage during the same year) do not overlap, as non-fed larvae die within a year (Yuval and Spielman 1990). Nymphs that are unable to feed on a suitable host during their first season of activity can overwinter and survive an additional season; thus, nymphal cohorts overlap (Yuval and Spielman 1990). If nymphs obtain a blood-meal before late summer (August), they will molt into adults by fall (Platt et al. 1992). However, if nymphs feed in late summer (August), they will overwinter (engorged) and molt to adults during the summer (late June or early July) of the subsequent year (Platt et al. 1992). A three-year life cycle has been proposed for *I. scapularis* in Michigan, U.S.A. (Hamer et al. 2012).

The life cycle of *I. scapularis* in southern Canada takes 3-4 years to complete (Lindsay et al. 1995, 1998). Hence, the duration of the life cycle is generally longer than that reported for blacklegged ticks in the Midwestern and Northeastern U.S.A, excluding those in Michigan (Hamer et al. 2012). Several factors, such as differences in habitat type, host availability, and climatic factors (e.g., temperature) influence the duration of the life cycle in southern Canada (Lindsay et al. 1995, 1998, 1999a, 1999b, Gatewood et al. 2009). For example, blacklegged ticks

are able to survive the prolonged periods of subzero temperatures and snowfall by seeking protection under leaf litter and entering a state of diapause (i.e., arrested development) (Lindsay et al. 1995, Belozerov and Naumov 2002). As temperatures increase, *I. scapularis* becomes active and resumes its development (Lindsay et al. 1995). In southern Canada (i.e., Ontario), females lay their eggs during late April or early May, regardless of whether they feed in November and then overwinter or whether they overwinter and then feed during April of the next year (Lindsay et al. 1995). Eggs hatch during mid-to-late July (Lindsay et al. 1995). Unfed adults that survive the winter tend to die by July (Lindsay et al. 1995). If larvae obtain a meal from a suitable host in mid-July or later in the year that they hatched, they will overwinter before molting to nymphs in the subsequent year (Lindsay et al. 1995). However, if larvae obtain a meal between late April and early July of the following year, they will molt to nymphs during that year (Lindsay et al. 1995). Similarly, if nymphs obtain a blood-meal between late April and early June, they will molt to adults during that year, whereas if they feed in late June or later, they will overwinter before molting to adults (Lindsay et al. 1995).

In Ontario, adult *I. scapularis* have two distinct periods of activity: mid-March to mid-June, and late September to early December (Lindsay et al. 1999a). Nymphs are most abundant in June and July, but are generally active from early May to late September (Lindsay et al. 1999b). Larvae are primarily active during mid-to-late June and also in mid-August; however, in some years, these peak periods of activity may be indistinct (Lindsay et al. 1999b). There is little to no overlap of larval cohorts, because *I. scapularis* larvae that overwinter tend to die before the second yearly peak of larval activity, which takes place in August (Lindsay et al. 1998, 1999b). There is also little overlap of larval cohorts of a given year and host-seeking nymphs, as nymphal activity peaks in June and July and larval cohorts emerge thereafter (Lindsay et al. 1999b). However, there is typically overlap between the seasonal host-seeking patterns of nymphs and of larvae that have overwintered (Lindsay et al. 1999b).

The seasonal activity patterns of *I. scapularis* nymphs in Ontario can be complicated, as unfed nymphs may survive for more than a year (Lindsay et al. 1998, 1999b). In general, however, the seasonal patterns of activity of immature *I. scapularis* in Ontario are probably similar to those of tick populations in Wisconsin, Connecticut, and Illinois, as well as in Massachusetts and New York (i.e., in the Midwest and Northeast, U.S.A., respectively) (Anderson and Magnarelli 1980, Daniels et al. 1989, Yuval and Spielman 1990, Siegel et al.

1991, Platt et al. 1992). Studies have shown that there is a high degree of seasonal synchrony of larval and nymphal *I. scapularis* in the Midwest, U.S.A., but little seasonal overlap in the Northeast, U.S.A. (Daniels et al. 1989, Platt et al. 1992, Gatewood et al. 2009, Hamer et al. 2012). Thus, there may be a geographical continuum of phenologies of *I. scapularis* (Hamer et al. 2012).

There are several biological, ecological, and genetic differences between *I. scapularis* in the Northeast and Midwest, U.S.A., and those in the Southeast, U.S.A. For example, in the south, seasonal activities of larval and nymphal *I. scapularis* appear to be unimodal and synchronous (Oliver et al. 1993a). In the north, two patterns have been observed: bimodal larval activity that is predominantly asynchronous with peak nymphal activity (e.g., Northeast and Midwest, U.S.A.), and unimodal larval activity that is synchronous with peak nymphal activity (e.g., Midwest, U.S.A.) (Lindsay et al. 1999b, Hamer et al. 2012). Furthermore, DNA sequence analyses of the mt large subunit and small subunit rRNA genes (i.e., the mt 16S and 12S rRNA genes, respectively) have shown that *I. scapularis* in the U.S.A. represent two genetically distinct groups: the "southern" and "American" clades (Rich et al. 1995, Norris et al. 1996, Qiu et al. 2002, Trout et al. 2009, Humphrey et al. 2010, Van Zee et al. 2013). Additionally, blacklegged ticks collected from the Northeast and Midwest, U.S.A. are frequently reported to bite humans, whereas those in the Southeast, U.S.A. are not (Falco and Fish 1988a, 1988b, Luckhart et al. 1992, Mitchell et al. 1996, Norris et al. 1996, Merten and Durden 2000). Host usage by immature *I. scapularis* also varies by region, despite identical host preferences in the laboratory (James and Oliver 1990, Keirans et al. 1996). For example, in the Northeast and Midwest, U.S.A., larval and nymphal blacklegged ticks parasitize *Peromyscus leucopus* (white-footed mouse) (Godsey et al. 1987, Adler et al. 1992, Stafford et al. 1995, Keirans et al. 1996, Walker et al. 1996). In contrast, *I. scapularis* immatures parasitize several species of lizards in the south (Oliver et al. 1993a, Keirans et al. 1996, Durden et al. 2002).

At least 125 species of vertebrates have been recorded as hosts for blacklegged ticks in the U.S.A. (Keirans et al. 1996). Adult *I. scapularis* have been recorded on 27 species of mammals and one species of lizard (Keirans et al. 1996). Important hosts of adult blacklegged ticks include *Odocoileus virginianus* (white-tailed deer), *Bos taurus* (cattle), and *Canis lupus familiaris* (dog) (Watson and Anderson 1976, Main et al. 1981, Wilson et al. 1984, Keirans et al. 1996, Riehle and Paskewitz 1996, Massung et al. 2005, Cortinas and Kitron 2006, Bouchard et

al. 2013, Lee et al. 2013). Adult *I. scapularis* have also been collected from several species of exotic animals living in Georgia and Florida, U.S.A., such as *Macaca silensus* (lion-tailed macaque), *Tragelaphus imberbis* (lesser kudu), and *Camelus dromedaries* (dromedary) (Keirans et al. 1996). Larvae and nymphs of *I. scapularis* have been recorded on 41 species of mammals, 14 species of lizards, and 57 species of birds (Keirans et al. 1996). These include rodents and small mammals, such as *P. leucopus*, *Napaeozapus insignis* (woodland jumping mouse), *Tamias striatus* (eastern chipmunk), *Tamiasciurus hudsonicus* (red squirrel), *Sciurus carolinensis* (eastern gray squirrel), *Clethrionomys gapperi* (southern red-backed vole), *Blarina brevicauda* (northern short-tailed shrew), *Sorex cinereus* (masked shrew), *Procyon lotor* (raccoon), *Didelphis virginiana* (Virginia opossum), *Mephitis mephitis* (striped skunk), and several species of migratory birds, such as *Dumetella carolinensis* (gray catbird), *Zonotrichia albicollis* (white-throated sparrow), *Catharus guttatus* (hermit thrush), *Seiurus aurocapillus* (ovenbird), *Cyanocitta cristata* (blue jay), *Troglodytes aedon* (house wren), *Geothlypis trichas* (common yellowthroat), *Oporornis agilis* (Connecticut warbler), and *Turdus migratorius* (American robin) (Piesman and Spielman 1979, Main et al. 1982, Godsey et al. 1987, Battaly and Fish 1993, Stafford et al. 1995, Keirans et al. 1996, Nicholls and Callister 1996, Hamer et al. 2010, 2011, 2012). Reptile hosts include *Eumeces* spp. (skinks) and *Ophisaurus* spp. (glass lizards) (Keirans et al. 1996, Kollars et al. 1999). In Canada, adult *I. scapularis* have been reported on *O. virginianus* (Watson and Anderson 1976, Barker et al. 1992, Lindsay et al. 1999a, Bouchard et al. 2013), while immature *I. scapularis* parasitize *P. leucopus*, *T. striatus*, *Peromyscus maniculatus* (North American deer mouse), and *T. hudsonicus*, as well as many species of passerine bird (e.g., *G. trichas*, *Parus atricapillus* (black-capped chickadee), *T. aedon*, *D. carolinensis*, *Catharus ustulatus* (Swainson's thrush), *Catharus minimus* (gray-cheeked thrush), *Melospiza lincolnii* (Lincoln's sparrow), and *Dendroica petchia* (yellow warbler)) (Lindsay et al. 1999b, Scott et al. 2001, Bouchard et al. 2011, Scott et al. 2012).

1.4.4. Pathogens

Blacklegged ticks are vectors of several pathogens of public health significance. These include: *B. burgdorferi*, the causative agent of Lyme disease; *A. phagocytophilum*, the agent of human granulocytic anaplasmosis; *Babesia microti*, the protozoan that causes human babesiosis; and the Powassan virus, which is the agent of Powassan encephalitis (McLean and Donohue

1959, McLean et al. 1961, McLean 1963, Spielman 1976, Ruebush et al. 1977, Spielman et al. 1979, Burgdorfer et al. 1982, Johnson et al. 1984, Bakken et al. 1994, Chen et al. 1994, Costero and Grayson 1996, Keirans et al. 1996, Hodzic et al. 1998, Estrada-Peña and Jongejan 1999). In the U.S.A., the reported annual incidences of Lyme disease and human granulocytic anaplasmosis have increased in recent years.

The modes of transmission of *A. phagocytophilum*, *B. burgdorferi*, and *B. microti* are similar, and differ from that of the Powassan virus (Davis and Bent 2011). For example, while there is transstadial transmission of *B. burgdorferi*, *A. phagocytophilum*, *B. microti*, and the Powassan virus within *I. scapularis* (Ebel and Kramer 2004, Davis and Bent 2011), transovarial transmission has been observed only for the Powassan virus (Costero and Grayson 1996, Davis and Bent 2011). Transovarial transmission may be important for maintaining a source of infection for vertebrate animals, because it allows for the pathogenic agents to survive environmental conditions that are fatal to adult ticks, but not eggs (Spickler and Roth 2008). Playing a larger and more important role in maintaining a source of infection for vertebrate hosts of *I. scapularis* than transovarial transmission is horizontal transmission, which, via systemic infection in the vertebrate host, is the most commonly recognized route of transmission of *B. burgdorferi*, *A. phagocytophilum*, and *B. microti* (Nonaka et al. 2010, Davis and Bent 2011, Hersh et al. 2012). Co-feeding transmission has also been documented for *B. burgdorferi*, *A. phagocytophilum*, *B. microti*, and the Powassan virus in *I. scapularis* (Davis and Bent 2011). This mode of transmission may also be important for maintaining a source of infection of these pathogens for animals, as the natural tendency of parasites (e.g., *I. scapularis*) to aggregate among and on their hosts facilitates the horizontal transmission of their associated pathogens (e.g., *B. burgdorferi* and the Powassan virus) (Watson and Anderson 1976, Shaw and Dobson 1995, Stafford et al. 1995, Randolph et al. 1996, Shaw et al. 1998, Tsao 2009, Nonaka et al. 2010, Bouchard et al. 2013). This facilitation is thought to occur, because greater numbers of co-feeding ticks on a host translates into a greater repression of the host immune system by tick saliva (Tsao 2009). Also, synchronized seasonal activity of the different life cycle stages of *I. scapularis* is thought to enhance the horizontal transfer of pathogens, including those between infected and uninfected co-feeding ticks (Tsao 2009).

Although the modes of transmission of *I. scapularis*-borne pathogens are similar (e.g., transstadial transmission of *B. burgdorferi*, *A. phagocytophilum*, *B. microti*, and the Powassan

virus and negligible to nonexistent transovarial transmission of *B. burgdorferi*, *A. phagocytophilum*, *B. microti*), the ways in which these pathogens are maintained in nature differs among them. For example, *B. burgdorferi* is a generalist microparasite exhibiting efficient interspecies transmission (i.e., a low degree of vertebrate host specialization) (Hanincova et al. 2006). Also, several species of passerine birds are suspected reservoir hosts of *B. burgdorferi* (e.g., *Thryomanes bewickii* (Bewick's wren), *G. trichas*, *Junco hyemalis* (dark-eyed junco), *Agelaius phoeniceus* (red-winged blackbird), *Melospiza melodia* (song sparrow), *C. ustulatus*, *Melospiza georgiana* (swamp sparrow), *and Z. albicollis*) (Scott et al. 2012). Furthermore, although *P. leucopus* and *O. virginiansus* are important hosts of *I. scapularis* (Keirans et al. 1996), neither is implicated as an overwintering reservoir host for *B. burgdorferi* (Lindsay et al. 1997, Tsao 2009). This is because the period of *B. burgdorferi* infectivity in *P. leucopus* is short (i.e., >7, but <21 days; Lindsay et al. 1997), *O. virginianus* are primarily parasitized by adult blacklegged ticks (Keirans et al. 1996), and *B. burgdorferi* is not transovarially transmitted by *I. scapularis* (Rollend et al. 2013). Additionally, most of the known genotypes (i.e., sequence types) based on a portion of the *rrs* (16S)–*rrl* (23S) intergenic spacer of *B. burgdorferi* can infect a wide range of rodent hosts and several genotypes can infect up to five host species (Hanincova et al. 2006). Moreover, *P. leucopus* is capable of harbouring multiple outer surface protein C (*ospC*) variants of *B. burgdorferi* over an entire transmission season (Swanson and Norris 2008). Similarly, *B. microti* infects a wide range of vertebrate hosts, although the reservoir competency of its vertebrate hosts differs (Hersh et al. 2012). For example, *P. leucopus* possesses greater reservoir competency of *B. microti* than several other small mammals and raccoons, the latter of which have comparable reservoir competencies (Hersh et al. 2012). Little is currently known regarding the extent of the genetic variation in *B. microti*; thus, knowledge of the strains transmitted by its different hosts is lacking.

In contrast, there is some evidence that *A. phagocytophilum* is host-specific, based on phylogenetic analysis of the different genotypes of the ankyrin (*ankA*) gene (Scharf et al. 2011). Furthermore, *P. leucopus*, *O. virginiansus*, *P. lotor*, *S. carolinensis*, *T. striatus*, and *Capra* spp. (goats) have also been shown to be competent reservoirs of *A. phagocytophilum*, while *T. migratorius* and *D. carolinensis* are not particularly susceptible to *A. phagocytophilum*, do not become bacteremic when exposed to *A. phagocytophilum*, and, thus, are not reservoir-competent hosts for *A. phagocytophilum* (Telford et al. 1996, Levin et al. 2002, Massung et al. 2002, 2003,

2005, 2006, Johnston et al. 2013). Additionally, strains of *A. phagocytophilum* differ in their abilities to infect vertebrate hosts. For example, a strain (i.e., Ap-variant 1) of *A. phagocytophilum* based on DNA sequences of a portion of the 16S rRNA gene that is not currently associated with human infection (Chen et al. 1994, Massung et al. 1998) is harboured by *O. virginiansus* (Massung et al. 2005), but not by *P. leucopus*, *Mus musculus* (house mouse), *T. striatus*, or *P. lotor* (Massung et al. 2003b, Yabsley et al. 2008). On the contrary, the 16S rRNA gene strain (i.e., Ap-ha) of *A. phagocytophilum* that has been associated with human infection (Chen et al. 1994, Massung et al. 1998), as well as infection in *P. leucopus* and *T. striatus* (Massung et al. 2002), has not been detected in (and is unable to persistently infect) *O. virginiansus* (Massung et al. 2005, Michalski et al. 2006, Reichard et al. 2009). Furthermore, the strains of *A. phagocytophilum* (e.g., Ap-ha and Ap-variant 1) differ in frequency among geographical regions (Carter et al. 2001, Bacon et al. 2008, Dahlgren et al. 2011, Margos et al. 2012).

The maintenance of *B. burgdorferi* in nature is thought to be the result of horizontal transmission between infected nymphal ticks of one cohort and uninfected larval ticks of another through a vertebrate host (Tsao 2009). This process may occur via two possible transmission cycles: 1) between nymphs that become infected from hosts during spring/summer and uninfected larvae in summer, or 2) between nymphs that become infected from hosts in autumn and uninfected larvae in the spring/summer of the following year (Ogden et al. 2008a). The maintenance of *A. phagocytophilum* and *B. microti* in nature is less well understood, but is probably also the result of horizontal transmission between infected and uninfected *I. scapularis* through a vertebrate host, since evidence that *A. phagocytophilum* and *B. microti* are transmitted transovarially within *I. scapularis* populations is lacking (Massung et al. 2005, Davis and Bent 2011, Hersh et al. 2012). A combination of high systemic host infection, intermediate tick co-feeding, and low vertical transmission rates can maintain the Powassan virus (Nonaka et al. 2010). When vertical transmission is rare, nymphal *I. scapularis* play an important role in the maintenance of this virus in nature (Nonaka et al. 2010).

There is evidence that the natural transmission cycles of *B. burgdorferi* and *A. phagocytophilum* are independent of one another, as infection with one of these pathogens does not hamper the acquisition of the other (Pancholi et al. 1995, Schwartz et al. 1997, Schauber et al. 1998, Courtney et al. 2003, Schulze et al. 2005, Steiner et al. 2008). There are also differences

in terms of the period (or duration) of infectivity in rodents of *B*. *burgdorferi* (>7 days to life; Lindsay et al. 1997, Oliver et al. 2003), *A*. *phagocytophilum* (14-21 days; Levin and Ross 2004), and the Powassan virus (2-3 days; Nonaka et al. 2010).

1.4.5. Population genetics and phylogeography

The population genetics and phylogeographical patterns of *I*. *scapularis* in the U.S.A. have been examined using both nDNA markers (e.g., ixolaris 2A, defensin, heat shock protein 70 b2, tick receptor for the *Borrelia burgdorferi* outer surface protein A (*TROSPA*), ferredoxin-glutamate synthase, dihydropyrimidine dehydrogenase, serotonin 4 receptor, acetylcholinesterase, and cytochrome c oxidase polypeptide) and mtDNA markers (i.e., 16S and 12S rDNA) (Rich et al. 1995, Norris et al. 1996, Qiu et al. 2002, Foley et al. 2008a, Trout et al. 2009, Humphrey et al. 2010, Van Zee et al. 2013). In general, significant genetic structure has been detected among populations of *I*. *scapularis* within the Midwest and/or Northeast when compared to the Southeast (Rich et al. 1995, Norris et al. 1996, Qiu et al. 2002, Foley et al. 2008a, Humphrey et al. 2010, Van Zee et al. 2013). This suggests that, historically, gene flow among tick populations between these two geographical areas was restricted (i.e., that *I*. *scapularis* populations suffered significant barriers to gene flow for an extended period of time in the past) (Humphrey et al. 2010). Conversely, due to phylogeographical similarity, some of these studies have failed to detect a significant genetic structure among populations of *I*. *scapularis* between the Midwest and Northeast regions (Norris et al. 1996, Humphrey et al. 2010). This suggests that, historically, there were no significant barriers to gene flow among populations of *I*. *scapularis* in the Midwest, as compared to the Northeast (Humphrey et al. 2010). However, estimations of time to the most recent common ancestor for tick populations in each of these three geographical areas have revealed that tick populations in the Midwest are younger than those in the Northeast, which are both younger than those in the Southeast (Humphrey et al. 2010). Thus, there is some evidence of genetic divergence among tick populations in the Midwest, as compared to the Northeast (Humphrey et al. 2010). In contrast to this historical gene flow, current gene flow among *I*. *scapularis* populations within and among geographical regions in the U.S.A. has been found to be restricted, even among neighbouring sites within regions (Norris et al. 1996, Qiu et al. 2002, Trout et al. 2009, Humphrey et al. 2010).

Two studies have investigated the population genetics of *I. scapularis* in southern Canada (Krakowetz et al. 2011, Mechai et al. 2013). In the study by Krakowetz et al. (2011), analyses of the mt 16S rRNA gene showed that all blacklegged ticks in southern Canada belonged to the "American" clade, as defined by Norris et al. (1996). Furthermore, despite small sample sizes, Krakowetz et al. (2011) showed that there were significant genetic differences between geographically isolated populations in Manitoba, Ontario and Nova Scotia. This suggested that the established populations in southern Canada may have been derived from different geographical areas in the U.S.A. (Krakowetz et al. 2011). In addition, these different geographical origins may be related to the different flyways used by migratory passerine birds transporting larval and nymphal ticks into Canada (Krakowetz et al. 2011). Subsequently, Mechai et al. (2013) examined the genetic variability in the mt cytochrome c oxidase subunit I gene of *I. scapularis* collected in Canada as part of a nationwide passive surveillance program. They found significant differences in the frequencies of haplotypes (i.e., sequence types) among four geographical regions: 1) western provinces, 2) eastern Ontario, 3) Quebec, and 4) Atlantic Provinces (Mechai et al. 2013), providing support for the hypothesis that the *I. scapularis* in different regions of Canada have originated from colonizing individuals in different geographical areas in the U.S.A (Mechai et al. 2013). However, Krakowetz et al. (2011) recommended that larger samples sizes and multiple genetic markers are needed to test this hypothesis.

1.5. Research objectives

Given that the distributional range of *I. scapularis* is predicted to expand within southern Canada (Ogden et al. 2005, 2006a, 2006b, 2008a, 2008b, 2008c, 2008d, Leighton et al. 2012, Ogden et al. 2013b), and that there is geographical variation in the U.S.A. of the prevalences of human diseases caused by the pathogens that this tick species carries (Bacon et al. 2008, Dahlgren et al. 2011), a key question needs to be addressed—namely, where are the geographical sources in the U.S.A. of the blacklegged ticks that occur in southern Canada? Answering this question is important for assessing the potential risk of exposure to pathogens carried by *I. scapularis* for humans and domestic animals living in different geographical areas of Canada. Therefore, the aim of my Ph.D. research was to examine the population genetics and phylogeographical patterns of *I. scapularis* and *A. phagocytophilum* vectored by *I. scapularis*, using multiple genetic markers, to determine the geographical origins of blacklegged ticks in

Canada. Based on the published information on *I. scapularis* and its pathogens, I proposed to test the following hypotheses:

1) that the genotypes/haplotypes of *I. scapularis* in Ontario and other eastern provinces will differ markedly from those in Manitoba, Saskatchewan, and Alberta (i.e., western Canada),
2) that the genotypes/haplotypes of *I. scapularis* in the Midwest, U.S.A. (i.e., Minnesota) will differ markedly from those present in the Northeast, U.S.A. (i.e., Rhode Island),
3) that there will be greater genetic similarity of *I. scapularis* populations in Manitoba to those in the Midwest, U.S.A., than to populations in eastern Canada or the Northeast, U.S.A.,
4) that there will be greater genetic similarity of *I. scapularis* populations in Ontario to those in the Northeast, U.S.A., than to populations in western Canada or the Midwest, U.S.A.,
5) that the phylogeographical patterns of *A. phagocytophilum* will mirror those of *I. scapularis*, and
6) that the combined phylogeographical patterns of *I. scapularis* and *A. phagocytophilum* will indicate their geographical origins in the U.S.A.

Hence, in this thesis, I examined the genetic diversity, population genetics, and phylogeography of *I. scapularis* from established populations in southern Canada, Minnesota, and Rhode Island using several mitochondrial and nuclear genes as genetic markers (Chapters 2-6). In addition, studies were conducted to determine if there were geographical differences in the presence of different strains of *A. phagocytophilum* within *I. scapularis*. Specifically, in Chapter 7, PCR-based assays were designed targeting the 16S rRNA gene to distinguish the human pathogenic strain of *A. phagocytophilum* in *I. scapularis* from a strain that is not associated with human disease in the different geographical areas. Then, the phylogeographical relationships of *A. phagocytophilum* were examined (Chapter 8) to determine whether this bacterium and its arthropod vector share a common evolutionary or biogeographic history. In the general discussion (Chapter 9), I compared the phylogeographical patterns of *I. scapularis* and *A. phagocytophilum* to determine the potential geographical origins of blacklegged ticks in Canada.

CHAPTER 2
GENETIC VARIATION IN THE MITOCHONDRIAL 16S RIBOSOMAL RNA GENE OF *IXODES SCAPULARIS* FROM DIFFERENT REGIONS OF CANADA AND THE U.S.A.[1]

2.1. Abstract

The DNA sequences of Domains IV and V of the mitochondrial (mt) 16S ribosomal (r) RNA gene were determined for 582 *Ixodes scapularis* collected from 11 geographical regions in Canada and the U.S.A. Mutational changes in the DNA sequences among ticks were examined in relation to their position in the predicted secondary structure of the mt 16S rRNA. A total of 52 sequence variants (i.e., haplotypes) of *I. scapularis* were detected among the geographical areas studied, which was 2-7 times greater than the number of haplotypes found in any study thus far. Estimates of haplotype richness suggest that the number of haplotypes detected in some of the geographical areas may still represent an underestimation of the genetic variability in the mt 16S rRNA gene for *I. scapularis*. Mutational differences were detected at 42 positions in the alignment of the 52 haplotypes, many of which occurred within a "hypervariable region" of the mt 16S rRNA gene. There were significant differences in genetic diversity among established populations of *I. scapularis*, and there was an association between genetic (F_{ST}) and geographical (km) distances between pairs of populations. There was also an association between some tick haplotypes and geographical areas in Canada, supporting the hypothesis of different U.S.A. origins for these ticks.

2.2. Introduction

The 3' end (i.e., Domains IV and V) of the mitochondrial (mt) 16S ribosomal (r) RNA gene has been used as a molecular marker to examine the population genetics of a variety of animals including chordates (e.g., Kanthaswamy and Smith 2004, Hafner et al. 2008, Harris and Perera 2009, Perera et al. 2012), molluscs (e.g., Pinceel et al. 2005), annelids (e.g., Minamiya et

[1] Part of this chapter was reproduced with permission from BioMed Central (http://www.biomedcentral.com/about/copyright/): **Krakowetz CN, Lindsay LR, Chilton NB (2014)** Genetic variation in the mitochondrial 16S ribosomal RNA gene of *Ixodes scapularis* (Acari: Ixodidae). Parasit Vectors 7. NBC conceived the project. LRL organized the collection of samples. CNK carried out laboratory work. CNK and NBC performed the data analyses. All authors interpreted the data, wrote the manuscript, and approved the final manuscript.

al. 2009), and arthropods (e.g., France and Kocher 1996, Austin et al. 2005, Szalanski et al. 2008, Liu et al. 2011). Sequence variation in this gene has also been examined for several species of ixodid ticks (e.g., Teglas et al. 2005, Foley et al. 2008a, Ketchum et al. 2009, Patterson et al. 2009, Krakowetz et al. 2010, Noureddine et al. 2011), including the blacklegged tick, *Ixodes scapularis* (see Rich et al. 1995, Norris et al. 1996, Qiu et al. 2002, Trout et al. 2009, Humphrey et al. 2010, Krakowetz et al. 2011, Van Zee et al. 2013), the principal vector of *Borrelia burgdorferi* sensu stricto, the causative agent of Lyme disease in North America (Burgdorfer et al. 1982, Johnson et al. 1984). Blacklegged ticks are also important vectors of *Anaplasma phagocytophilum*, the etiologic agent of human granulocytic anaplasmosis (Bakken et al. 1994, Chen et al. 1994, Hodzic et al. 1998), *Babesia microti*, the causative agent of human and rodent babesiosis (Spielman 1976, Spielman et al. 1979, Keirans et al. 1996), and the Powassan (POW) virus, the etiologic agent of POW encephalitis (McLean and Donohue 1959, Costero and Grayson 1996, Kramer et al. 2013).

The distribution of *I. scapularis* in the U.S.A. encompasses 35 states and ranges from Texas, Oklahoma, and Kansas in the southwest Midwest to Florida, Georgia, South Carolina, and North Carolina in the Southeast, and from South Dakota, Minnesota (MN), Wisconsin (WI), and Iowa in the northern (i.e., Upper) Midwest to Maine, Vermont, New Hampshire, New York (NY), Massachusetts (MA), Connecticut (CT), Rhode Island (RI), and Pennsylvania (PA) in the Northeast (Dennis et al. 1998). Although the distribution of this species spans from the southwest Midwest across to the Southeast and up to the northeastern states, established populations of *I. scapularis* in the Upper Midwest are geographically isolated from other populations (Dennis et al. 1998).

Population genetics and phylogeographical studies based on analyses of the DNA sequences of the mt large subunit (LSU) and small subunit (SSU) rRNA genes (i.e., the mt 16S and 12S rRNA genes, respectively) have shown that *I. scapularis* in the U.S.A. can be divided into two major clades: the "southern" and "American" clades (Rich et al. 1995, Norris et al. 1996, Qiu et al. 2002, Trout et al. 2009, Humphrey et al. 2010, Van Zee et al. 2013). The DNA sequences of the mt 16S rRNA gene of ticks from the "southern" clade differ by at least 9 bp when compared to the sequences of individuals from the "American" clade (Qiu et al. 2002). In contrast, Qiu et al. (2002) showed that 17 haplotypes (i.e., sequence variants) of the "American" clade differed from one another by 1-6 bp, whereas three haplotypes of the "southern" clade

differed from one another by 3-6 bp. Blacklegged ticks of the "southern" clade have only been reported from southern states (i.e., North Carolina, South Carolina, Georgia, Oklahoma, Texas, Arkansas, and Florida), while those of the "American" clade occur throughout the distributional range of the species (Rich et al. 1995, Norris et al. 1996, Qiu et al. 2002, Trout et al. 2009, Humphrey et al. 2010, Van Zee et al. 2013).

Within the "American" clade, one mt LSU rRNA gene haplotype (i.e., haplotype F of Qiu et al. 2002 = haplotype 1 of Humphrey et al. 2010) is the most commonly detected haplotype of *I. scapularis* and represents the central haplotype in minimum spanning networks depicting the relationships of haplotypes of the "American" clade (Qiu et al. 2002, Humphrey et al. 2010). The networks of the "American" clade (Qiu et al. 2002, Humphrey et al. 2010) have a "star" pattern, as described by Qiu et al. (2002), where haplotypes differ from the central haplotype by one to four mutational steps. A "star" pattern in a network is indicative of a population undergoing expansion (e.g., Qiu et al. 2002, Humphrey et al. 2010). Several other frequently detected haplotypes (e.g., A and D of Qiu et al. 2002), which differ in sequence by 1 bp from the central haplotype, have represented a link to additional haplotypes (Qiu et al. 2002), or, in combination with their most closely related members, began to resemble the "star" pattern of the entire clade (Humphrey et al. 2010). In contrast, networks of the "southern" clade lack a central haplotype (Qiu et al. 2002, Trout et al. 2009, Humphrey et al. 2010), which is indicative of a population that has not grown in size for much of its evolutionary history (Qiu et al. 2002).

The first reported population of *I. scapularis* in Canada was in Long Point Provincial Park (LPPP), Ontario (ON) in the early 1970s (Watson and Anderson 1976). Since then, several populations of *I. scapularis* have recently become established in several provinces in southern Canada: Manitoba (MB), Ontario, New Brunswick (NB), and Nova Scotia (NS) (Barker and Lindsay 2000, Ogden et al. 2008b, Ogden et al. 2008d, Ogden et al. 2009). Several other populations have recently established, or are in the process of establishing, in southwestern Quebec (QC) (Bouchard et al. 2011, Koffi et al. 2012). There are also many reports of adventitious (e.g., bird-borne) ticks occurring in several regions of southern Canada (e.g., Ogden et al. 2006c, Ogden et al. 2010, Koffi et al. 2012, Mechai et al. 2013). It has been proposed that the distributional range of *I. scapularis* in southern Canada has expanded as a consequence of the continual transportation of larval and nymphal ticks from the U.S.A. by migratory passerines during their spring migration (Klich et al. 1996, Scott et al. 2001, Morshed et al. 2005, Ogden et

al. 2008b, Scott et al. 2010, Scott et al. 2012). An estimated 50 to 175 million blacklegged ticks are transported into Canada by migratory birds each year (Ogden et al. 2008b). The distributional range of *I. scapularis* in Canada is expected to expand further, because the predicted environmental changes associated with global warming will provide more suitable conditions for tick survival and reproduction (Ogden et al. 2005, Ogden et al. 2006b, Ogden et al. 2008a, Ogden et al. 2008e, Leighton et al. 2012, Ogden et al. 2013a).

Genetic variability in the 3' end of the mt 16S rRNA gene has already been examined for a small number of blacklegged ticks from southern Canada (Krakowetz et al. 2011). Krakowetz et al. (2011) showed that *I. scapularis* collected from six established populations in Canada belonged to the "American" clade and that there were 19 haplotypes among the 153 ticks examined. In addition, there were significant differences in the genetic structure among populations, even though sample sizes were relatively small (i.e., 17-26 individuals per population; Krakowetz et al. 2011). It was recommended that additional studies are needed using larger sample sizes to test the hypothesis that the ticks from different provinces/geographical regions in southern Canada (i.e., the Atlantic, Central, and Prairie Provinces) originated from different areas in the U.S.A. (Krakowetz et al. 2011). The objectives of this chapter were to compare the frequencies of the different mt LSU rRNA gene haplotypes of *I. scapularis* from different geographical regions in southern Canada and the northern Midwest and Northeast U.S.A. and to determine the locations of the mutational differences in DNA sequence in relation to the secondary structure of Domains IV and V of the mt 16S rRNA.

2.3. Materials and methods

2.3.1. Samples

A total of 582 *I. scapularis* were collected from hosts or the environment between 2000 and 2011 (Table 2.1 and Fig. 2.1). This included 70 adventitious ticks collected from nine provinces in Canada (Table 2.2) and 512 ticks that were collected by drag sampling using the methodology of Lindsay et al. (1999b) at nine localities, each of which represented an established population of *I. scapularis*, in Canada and the U.S.A. (Fig. 2.1). The four Canadian populations of *I. scapularis* were Pembina Valley Provincial Park (PVPP) and Stanley Trail (ST)

Table 2.1. The number of *I. scapularis* collected in different years and at different life cycle stages from the 11 geographical regions in North America.

Region	Collection year(s)	No. of nymphs	No. of females	No. of males	Total
Canada					
Prairie					
Alberta (AB)	2011	0	2	0	2
Saskatchewan (SK)	2009, 2010	0	6	0	6
Manitoba (MB)	2010, 2011	0	46	50	96
Central					
Ontario (ON)	2000, 2006-2009, 2011	0	102	64	166
Quebec (QC)	2005-2007, 2009-2011	0	21	1	22
Atlantic					
New Brunswick (NB)	2007, 2009-2011	0	6	0	6
Prince Edward Island (PE)	2006, 2007, 2011	0	3	0	3
Nova Scotia (NS)	2006-2011	0	11	0	11
Newfoundland (NL)	2011	0	2	0	2
U.S.A.					
Midwest					
Minnesota (MN)	2008	0	84	84	168
Northeast					
Rhode Island (RI)	2009, 2010	100	0	0	100
Total		100	283	199	582

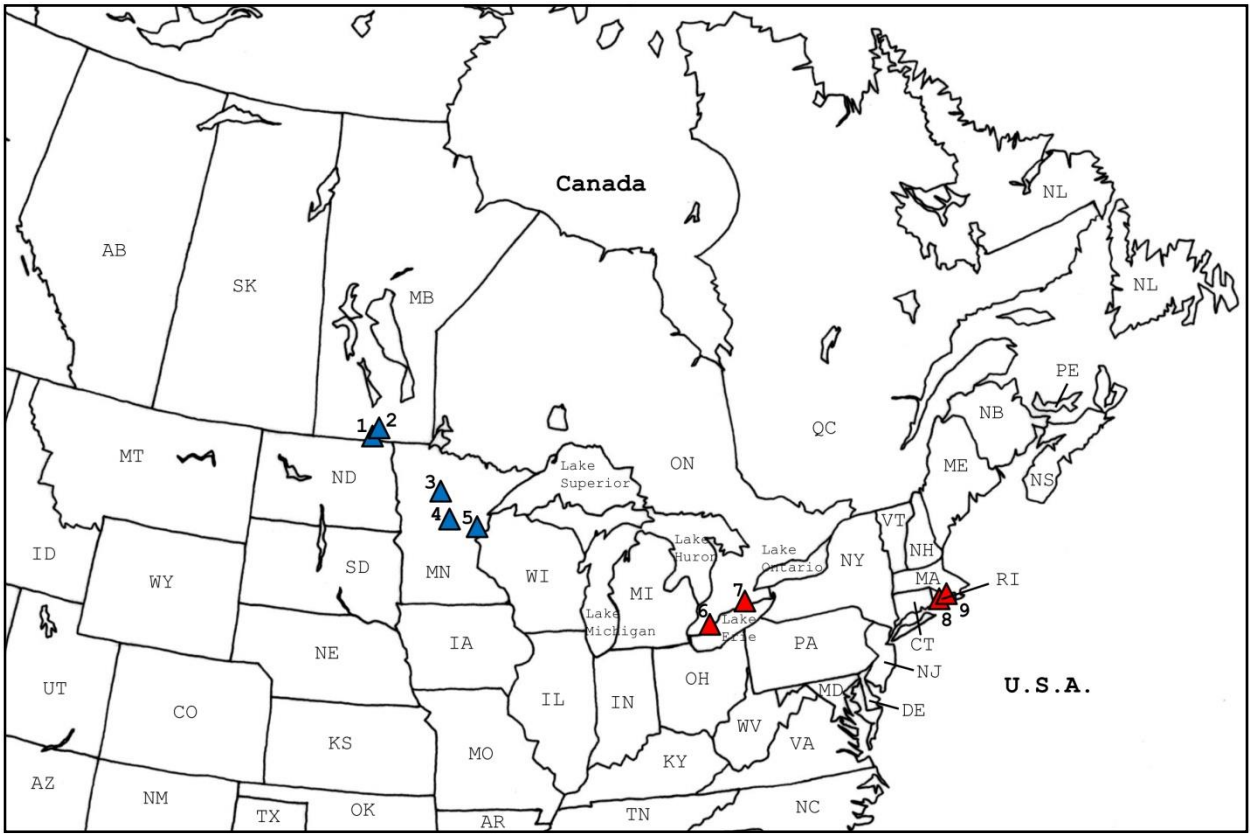


Figure 2.1. Localities in Manitoba (MB) and Ontario (ON), Canada and in Minnesota (MN) and Rhode Island (RI), U.S.A. from where adult and nymphal *I. scapularis* were collected for molecular analyses. Blue triangles represent established tick populations: 1) Pembina Valley Provincial Park, 2) Stanley Trail, 3) Itasca State Park, 4) Camp Ripley, and 5) St. Croix State Park in the "western" geographical area. Red triangles represent established tick populations: 6) Point Pelee National Park, 7) Long Point Provincial Park, 8) Trustom Pond, South Kingstown, and 9) Hazard Island, South Kingstown in the "eastern" geographical area.

Table 2.2. Collection details of the adventitious *I. scapularis* in Canada.

Year	Province*	*n*	Stage	Engorgement	Host	Travel history
2005						
	QC	2	F	partial	dog	local
2006						
	ON	2	F	partial	dog	local/Wisconsin**
	QC	3	F	partial	cat/dog	local
	PE	1	F	partial	dog	local
	NS	1	F	partial	dog	local
2007						
	ON	4	F	partial/unfed, attached	human/dog/unknown	local
	QC	2	F	partial	cat/dog	local
	NB	1	F	partial	cat	local
	PE	1	F	partial	dog	local
	NS	1	F	partial	dog	local
2008						
	ON	1	F	unfed, attached	human	local
	NS	2	F	partial/unfed, attached	human/cat	local
2009						
	SK	4	F	partial	dog	none
	MB	1	F	partial	dog	none
	ON	1	F	unfed	drag sample	Wainfleet Bog
	QC	2	F	partial/minimal	dog	local
	NB	1	F	unfed, attached	human	travel unknown
	NS	2	F	partial/minimal	dog	local
2010						
	AB	1	F	partial	dog	unknown
	SK	2	F	partial	dog	none/within province
	MB	2	F	partial	dog	none
	QC	6	F	partial/minimal	dog	local/New York state**
	NB	2	F	partial	dog	local
	NS	3	F	partial/minimal	human/dog	local/Florida**
2011						
	AB	1	F	partial	cat	local
	MB	3	F	full/partial/unfed,attached	human/dog	local
	ON	4	F	partial/minimal/unfed, attached	human/dog	local
	QC	7	F/M	partial/minimal/unfed, attached	cat/dog	local
	NB	2	F	partial/minimal	human	local
	PE	1	F	partial	dog	local
	NS	2	F	partial	cat/dog	local
	NL	2	F	partial/unfed, attached	human/dog	local
Total		70				

* AB = Alberta, SK = Saskatchewan, MB = Manitoba, ON = Ontario, QC = Quebec, NB = New Brunswick, PE = Prince Edward Island, NS = Nova Scotia, and NL = Newfoundland.
** Tick may have originated from an endemic area in the U.S.A.

in MB, and Point Pelee National Park (PPNP) and LPPP in ON, while the five established populations of *I. scapularis* in the U.S.A. were Itasca State Park (ISP), Camp Ripley (CR), and St. Croix State Park (CSP) in MN, and Trustom Pond (TPSK) and Hazard Island (HISK) in South Kingstown, RI.

2.3.2. Molecular analyses

The total genomic (g) DNA of each tick was extracted using the QIAamp DNA Mini Kit™ or the DNeasy Blood & Tissue Kit™ (Qiagen, Toronto, ON, Canada) as per the instructions of the manufacturer, except for the modifications described previously for each kit (Ogden et al. 2006b and Dergousoff and Chilton 2007, respectively). An ~400 bp region spanning Domains IV and V of the mt 16S rRNA gene was amplified by PCR from the total gDNA of each tick using the primers 16S−1 (5'-CTGCTCAATGATTTTTTAAATTGCTG TGG-3') and 16S+1 (5'-CCGGTCTGAACTCAGATCAAGT-3') (Norris et al. 1996) and the thermocycler conditions of Krakowetz et al. (2011). PCRs were performed in 25 µl or 50 µl reaction volumes comprised of 2.5 µl of 10X *Taq* Buffer with KCl (Fermentas, Thermo Fisher Scientific, Ottawa, ON, Canada), 1.75 mM $MgCl_2$ (Fermentas), 200 µM of each dNTP (Fermentas), 25 ρmol of each primer, 0.5 U and 1.25 U of *Taq* DNA Polymerase (Fermentas), respectively, and 1-2 µl of gDNA template. A negative (i.e., no gDNA) control was included in each set of PCRs. Amplicons were subjected to electrophoresis on 1.5% (w/v) agarose-TBE gels, as previously described by Krakowetz et al. (2011), prior to single-strand conformation polymorphism (SSCP) analyses, a mutation-scanning technique used to compare amplicons for sequence variability (Gasser et al. 2006). The methodology used for SSCP followed that of Krakowetz et al. (2011), except that 0.5-5 µl of the amplicons from each PCR were mixed with 0-4.5 µl of UltraPure™ water (Invitrogen™, Life Technologies, Burlington, ON, Canada) and 5 µl of loading buffer (Gel Tracking Dye™; Promega, Madison, U.S.A.). The resulting SSCP profiles (i.e., banding patterns) were reproducible when PCR-amplicons were run on the same or different gel(s) and day(s). Line-up gels were used to confirm the SSCP profiles of some samples. Amplicons (10 µl) representing the different banding patterns were transferred to sterile PCR tubes (Eppendorf Canada, Mississauga, ON, Canada) along with a 1 µl mixture containing 0.7 µl of 1X *Taq* Buffer with KCl (Fermentas), 3 U of exonuclease I (New England BioLabs, Whitby, ON, Canada), and 0.15 U of shrimp alkaline phosphatase (Fermentas) and purified for

15 min in a thermocycler set to 37ºC. To inactivate the enzymes, the temperature in the thermocycler was increased to 80ºC for 15 min. Automated DNA sequencing was conducted on 231 samples representing multiple amplicons of each SSCP profile type (where possible) using the primers 16S−1 and 16S+1 in separate reactions. The sequences of the different haplotypes have been deposited in GenBank™ under the accession numbers HG916768-HG916804.

2.3.3. Data analyses

Sequences were aligned manually, but modified according to the predicted secondary structure of Domains IV and V of the mt 16S rRNA that was constructed for *I. scapularis* based on the models of this rRNA for other arthropods (Gutell and Fox 1988, Gutell et al. 1993, Gutell 1996). For comparative purposes, a secondary structure model of the 3' end of the mt LSU rRNA was also constructed for a related species, *Ixodes ricinus*, using sequence data for 31 haplotypes (GenBank accession nos. GU074588-GU4647; Noureddine et al. 2011). Variable positions in the DNA sequence alignments of *I. scapularis* and *I. ricinus* were examined in relation to the predicted secondary structures for each species. The computer program PAUP v4.0b10 (Swofford 2002) was used to carry out a phylogenetic analysis using the neighbour-joining (NJ) method. The DNA sequence of Domains IV and V of the mt 16S rRNA gene of *I. pacificus* (GenBank accession no. AF309008) was used as the outgroup. A total of 1000 bootstrap replicates were used to test the support for the different branches in the NJ tree. The computer program TCS version 1.21 (Clement et al. 2000) was used to generate a minimum spanning network tree depicting the relationships of the haplotypes detected in this study (Tables 2.3 and 2.4). Also included in this analysis were some of the mt 16S rRNA gene haplotypes reported in other studies (Caporale et al. 1995, Rich et al. 1995, Norris et al. 1996, Qiu et al. 2002, Anstead and Chilton 2011, Krakowetz et al. 2011; see Table 2.4). The computer program Arlequin version WINARL35 (Excoffier and Lischer 2010) was used to determine the haplotype (*h*) and nucleotide (π) diversities of *I. scapularis* from the established populations where sample sizes were reasonably large (i.e., $n > 40$). Nucleotide diversity represents the average number of nucleotide differences per site for a group of DNA sequences, while haplotype diversity describes the probability that two randomly chosen ticks will not be of the same haplotype (Nei and Kumar 2000). The *D* statistics of Tajima (1989) and the F_S statistics of Fu (1997) were also

Table 2.3. The number of *I. scapularis* of the different mt 16S rRNA gene haplotypes (HT), and the variable positions in the aligned DNA sequences. A dot (.) at an alignment position indicates the same nucleotide as in the sequence of haplotype Is-1, while a dash (-) represents a gap.

| HT | *n* | *n** | Alignment position: | | |

Table 2.4. Haplotype designations used in the minimum spanning network tree of the present study in relation to those (i.e., haplotype, specimen no., or GenBank accession no.) used in previous studies conducted in Canada and the U.S.A.

Present study[a]	Krakowetz et al. 2011	Anstead and Chilton 2011	Qiu et al. 2002	Norris et al. 1996	Rich et al. 1995[b]	Caporale et al. 1995[c]
Is-1	Is-1	FR854227	Hap F	L43862, L43875	U26605_IL94[d]	U14145_DAM1, U14146_DAM2
Is-2	Is-2		Hap A		U26612_ME92b	
Is-3	Is-3		Hap C			
Is-4	Is-4		Hap D	L43857	U26613_MS93	
Is-5	Is-5		Hap E			
Is-6	Is-6		Hap G			
Is-7	Is-7		Hap H		U26600_CT93, U26608_MA92c	
Is-8	Is-8	FR854230[e]			U26617_NC94	
Is-9	Is-9				U26611_ME92a	
Is-10	Is-10			L43867		
Is-11	Is-11			L43873		
Is-12	Is-12					
Is-13	Is-13	FR854228				
Is-14	Is-14					
Is-15	Is-15					
Is-16	Is-16					
Is-17	Is-17					
Is-18	Is-18					
Is-19	Is-19					
Is-20			Hap B			
Is-21			Hap I			
IS-22			Hap J			
Is-23			Hap K			
Is-24			Hap L			
Is-25			NC1_6			
Is-26			NC2_22			
Is-27			NC2_29			
Is-28			NY2_11		U26600_CT93, U26608_MA92c	
Is-29			MA1			
Is-30				L43858		
Is-33				L43868		
Is-34				L43869		
Is-35				L43870		
Is-36				L43871, L43872		
Is-48					U26612_ME92b	
Is-49					U26600_CT93, U26608_MA92c	
Is-50 to Is-79						
Is-80			Hap M	L43855, L43856		
Is-81			Hap N			
Is-82			Hap O			

Table 2.4. Continued.

Present study[a]	Krakowetz et al. 2011	Anstead and Chilton 2011	Qiu et al. 2002	Norris et al. 1996	Rich et al. 1995[b]	Caporale et al. 1995[c]
Is-83				L43854[e]		
Is-84				L43861		
Is-85				L43863		
Is-86				L43865		
Is-87				L43866		

[a] Ticks in this study comprised haplotypes Is-1 to Is-10, Is-12 to Is-15, Is-17, Is-20 to Is-21, Is-23 to Is-24, Is-30, and Is-48 to Is-79.
[b] Based on sequences of only ≈342 bp (i.e., missing 18 bp at 5′ end and 51 bp at 3′ end).
[c] Based on sequences of only ≈341 bp (i.e., missing 18 bp at 5′ end and 52 bp at 3′ end).
[d] Also includes U26606_Ma92a, U26607_Ma92b, U26609_MD94, U26610_NJ92, U26615_NC93b, U26616_NC93c, U26618_WI92a, U26619_WI92b, U26620_W193, and U26621_NY91.
[e] Also includes L43864, L43874, L43876, and L43877.

determined using Arlequin. Under the neutral model, D and F_S values should be approximately zero. Significantly negative D ($p < 0.05$) and F_S ($p < 0.02$) values are indicative of populations undergoing expansion, whereas significantly positive values are characteristic of populations undergoing bottlenecks (Aris-Brosou and Excoffier 1996). Arlequin was also used to conduct a Chakraborty's test (1990), which relates a measure (i.e., F_{ST} value) of genetic differentiation between each pair of populations, and a Mantel test (using 1000 permutations), which determines if there was a correlation between genetic (F_{ST}) and geographical (km) distances.

Interpolation (i.e., rarefaction) curves and their 95% confidence intervals based on individual-based abundance data (i.e., haplotype frequency data) for each established population were generated using the computer program EstimateS (Colwell 2013) to estimate the number of haplotypes that would be expected when between one and n ticks are "collected" (i.e., characterized based on their sequences of the mt 16S rRNA gene), where n is the total number of ticks from each established population (e.g., 56 for ISP or 108 for LPPP). These curves were extrapolated by a factor of two using the same program to estimate the number of haplotypes to be expected when between n and $2n$ ticks are "collected" (or characterized). According to Colwell et al. (2012), extrapolations are most reliable to a doubling of sample size. The rarefaction curve for TPSK could not be extrapolated, as singletons were absent from the data set. The total haplotype richness based on progressively larger numbers of ticks between one and n from the data set for each established population was estimated (EstimateS) using the bias-corrected version of Chao 1 and 1000 runs (or randomizations). Rarefaction and extrapolation curves and their respective 95% confidence intervals based on sample-based abundance data (i.e., haplotype frequency data) from the four established populations in the east (i.e., PPNP, LPPP, TPSK, and HISK) and the five in the west (i.e., PVPP, ST, ISP, CR, and CSP) were also generated using EstimateS. The data from each region (i.e., "eastern" or "western") was input as a single set of replicated sampling units, so that Chao 2, a non-parametric estimator of species (or haplotype) richness, could be used (bias-corrected version; 1000 runs) to estimate the total haplotype richness based on successively larger numbers of ticks from the data set. Chao 2, which requires only presence/absence data, outperforms other non-parametric estimators based on small sample sizes, including Chao 1 (Colwell and Coddington 1994). Rarefaction and extrapolation curves and their respective 95% confidence intervals were also generated using EstimateS based on sample-based abundance data from the nine established populations. The

classic version of Chao 2 (1000 runs) was used to estimate haplotype richness for these data. For comparative purposes, a scatter plot of the number of haplotypes observed in each established population as a function of the total number of ticks from that population was created.

A hierarchical Analysis of Molecular Variance (AMOVA) test was conducted using Arlequin to estimate the genetic diversity within and among populations in different geographical regions. For this analysis, the nine established populations of *I. scapularis* were divided into four groups based on the provinces (Canada) or states (U.S.A.) in which they were located.

2.4. Results

Amplicons of the mt 16S rRNA gene derived from each tick (n = 582) produced a single band of the expected size (~450 bp) on agarose gels when subjected to electrophoresis (not shown). No bands were detected on agarose gels for the negative control samples (also not shown). There were 52 different SSCP profiles found among the 582 amplicons (Table 2.3). Amplicons with same SSCP profile were identical in DNA sequence, while those that differed in SSCP profile also differed in sequence by one or more base pairs. The 52 different SSCP profiles corresponded to 52 different mt LSU rRNA gene haplotypes, 22 of which had been reported previously in other studies (Caporale et al. 1995, Rich et al. 1995, Norris et al. 1996, Qiu et al. 2002, Anstead and Chilton 2011, Krakowetz et al. 2011; see Table 2.4). The DNA sequences of the mt 16S rRNA gene of multiple individuals of the different haplotypes varied in length from 404-407 bp. Sequences were aligned over 408 base pairs (Table 2.3). Those DNA sequences representing each of the 52 haplotypes differed from one another by 1-5 bp (Table 2.3). Genetic variation among haplotypes was detected at 42 alignment positions (Table 2.3). These mutational differences consisted of 18 purine transitions, seven pyrimidine transitions, 12 transversions, four indels, and one multiple mutational change. The locations of these mutational changes in relation to the predicted secondary structure of the 3' end of the mt 16S rRNA for *I. scapularis* are shown in Fig. 2.2. Twenty-six (61.9%) of the mutational changes occurred at unpaired sites in the secondary structure (e.g., end loops), while 11 (alignment positions: 57, 90, 151, 164, 193, 237, 258, 333, 364, 365, and 373; Fig. 2.2 and Table 2.3) represented partial-compensatory base pair changes that maintained the secondary structure of the rRNA. The location of the genetic variation in the 52 DNA sequences was not evenly distributed across the gene. The hypervariable

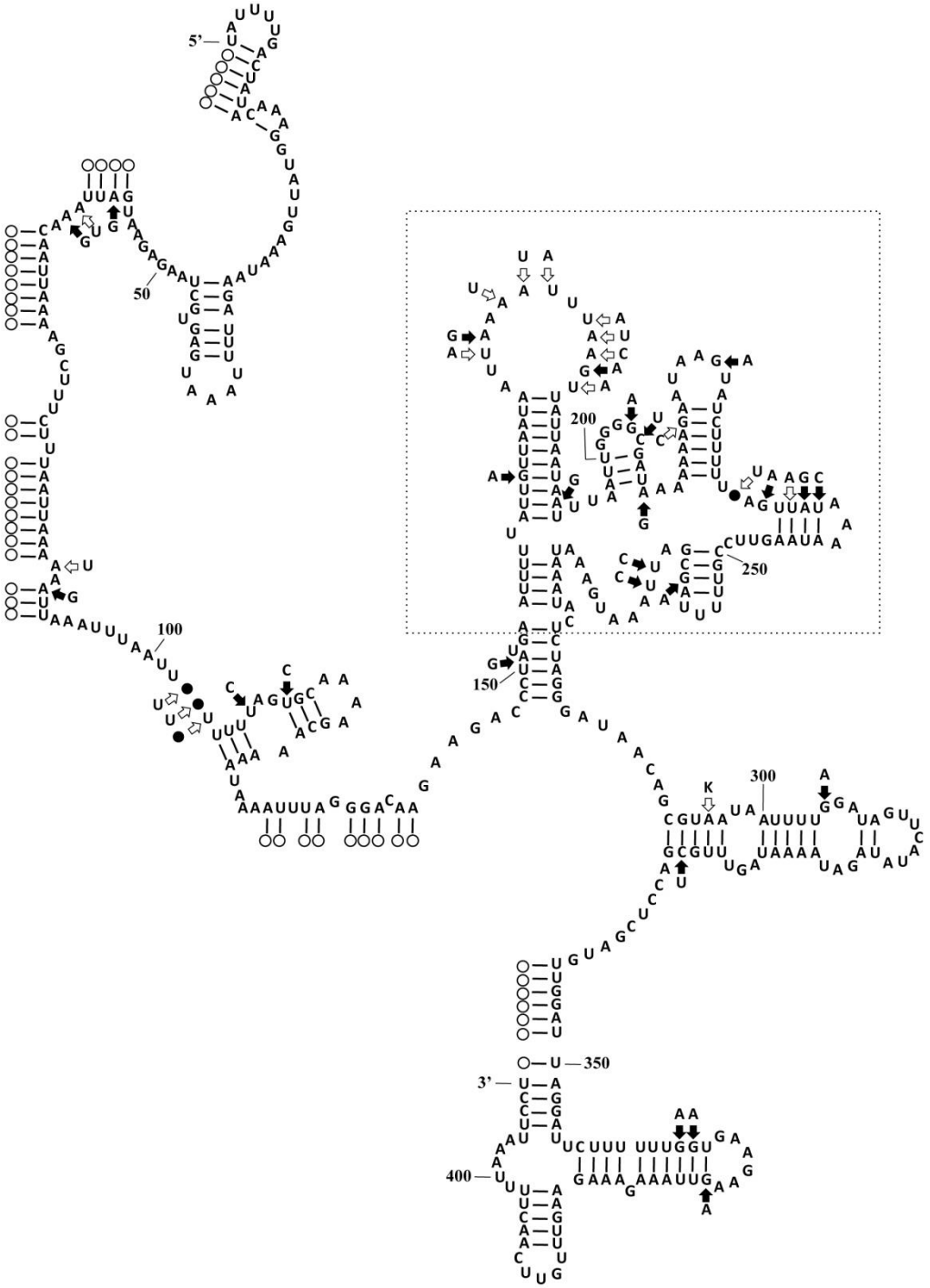


Figure 2.2. The secondary structure of the mt 16S rRNA for *I. scapularis* according to the models of Gutell and Fox (1988), Gutell et al. (1993), and Gutell (1996). Open circles indicate putative nucleotides within other regions of the mt 16S rRNA (see Gutell and Fox 1988; Gutell et al. 1993; Gutell 1996). Solid arrows indicate transition mutations, while open arrows indicate transversion, multiple, or insertion/deletion mutations relative to haplotype Is-1 in the 408 bp alignment of the haplotypes detected in this study. The box represents the hypervariable region (e.g., Black and Piesman 1994; Misof et al. 2002, Smith and Bond 2003).

region (i.e., alignment positions 154 to 279; Fig. 2.2) comprised only 126 (30.9%) of the 408 bp at the 3' terminal end of the mt 16S rRNA gene, but contained 25 (59.5%) of the 42 variable positions among the haplotypes. The proportion of variable positions in the hypervariable region was 19.8% (25 variable positions over 126 bp). In contrast, there was a significantly ($\chi^2_1 = 17.99$, $p < 0.001$) lower proportion of variable positions (6.0%; 17 variable positions over 282 bp) in the 5' and 3' regions flanking the hypervariable region, which represented 282 (69.1%) of the 408 bp at the 3' terminal end of the gene. These two flanking regions contained 17 (40.5%) of the 42 variable positions among sequence types.

Table 2.5 shows the number of *I. scapularis* from the different geographical regions of each haplotype of the mt 16S rRNA gene. The most common haplotype (i.e., haplotype Is-1) comprised 282 (48.5%) of the ticks collected, while another six haplotypes (i.e., haplotypes Is-2, Is-4, Is-7, Is-13, Is-15, and Is-63) comprised 173 (29.7%) of all ticks. Haplotype Is-1 was found in most Canadian provinces, except for NB and Newfoundland (NL); however, sample sizes from these provinces ($n = 6$ and $n = 2$, respectively) were small. Fifteen (28.8%) of the 52 haplotypes were each represented by a single tick (i.e., singletons). Furthermore, thirty-three (63.5%) of the haplotypes were each found in a single geographical area only. Haplotype Is-4 was found in eight of the 11 geographical regions sampled from, and haplotype Is-7 was detected in six regions including ON, QC, RI, NB, NS, and NL. In contrast, haplotype Is-15 was detected in ON only. Haplotype Is-2 was detected in seven provinces and/or states including MB, MN, ON, QC, RI, NB, and NS, whereas haplotype Is-13 was found in the three provinces of Saskatchewan (SK), MB, and ON, and in the state of MN.

The number of haplotypes detected in each of the nine established populations of *I. scapularis* in Canada and the U.S.A. varied from seven (TPSK, RI and PPNP, ON) to 14 (CR and CSP, MN) (Fig. 2.3). The only haplotype found in all nine established populations of *I. scapularis* was haplotype Is-1, which comprised 34% (HISK, RI) to 62.5% (ISP, MN) of the ticks examined from each locality. The second most common haplotype differed among the established populations (e.g., haplotype Is-15 in LPPP and haplotype Is-7 in HISK), and comprised between 7.1-17.4% of the ticks sampled in each population, except for LPPP (ON) and HISK (RI) where they comprised 22.2% and 32%, respectively.

The haplotype and nucleotide diversities of *I. scapularis* within the nine established populations ranged from 0.5994 to 0.7856 and 0.001911 to 0.003571, respectively (Table 2.6).

Table 2.5. The number of *I. scapularis* of the different mt 16S rRNA gene haplotypes collected from nine provinces* in Canada and two states (MN and RI) in the U.S.A.

Haplotype	*n*	No. individuals from:										
		AB	SK	MB	MN	ON	QC	RI	NB	PE	NS	NL
Is-1	282	1	2	46	100	77	8	41	0	1	6	0
Is-2	25	0	0	6	2	2	1	12	1	0	1	0
Is-3	1	0	0	0	0	0	1	0	0	0	0	0
Is-4	44	0	1	14	12	2	3	9	0	2	0	1
Is-5	3	0	0	0	0	0	0	3	0	0	0	0
Is-6	14	0	0	1	5	1	4	1	1	0	1	0
Is-7	33	0	0	0	0	5	1	24	1	0	1	1
Is-8	3	0	0	1	2	0	0	0	0	0	0	0
Is-9	11	0	0	4	3	4	0	0	0	0	0	0
Is-10	2	0	0	0	0	2	0	0	0	0	0	0
Is-12	2	0	0	0	0	0	0	2	0	0	0	0
Is-13	24	0	1	2	7	14	0	0	0	0	0	0
Is-14	3	0	0	0	3	0	0	0	0	0	0	0
Is-15	27	0	0	0	0	27	0	0	0	0	0	0
Is-17	1	0	0	0	1	0	0	0	0	0	0	0
Is-20	5	0	0	0	0	3	0	0	1	0	1	0
Is-21	1	0	0	0	0	0	1	0	0	0	0	0
Is-23	2	0	0	1	1	0	0	0	0	0	0	0
Is-24	2	0	0	0	0	0	1	0	0	0	1	0
Is-30	2	0	0	2	0	0	0	0	0	0	0	0
Is-48	2	0	0	0	2	0	0	0	0	0	0	0
Is-49	1	0	0	0	0	0	0	1	0	0	0	0
Is-50	3	0	0	0	0	3	0	0	0	0	0	0
Is-51	4	0	0	3	1	0	0	0	0	0	0	0
Is-52	4	0	0	2	2	0	0	0	0	0	0	0
Is-53	2	0	0	1	1	0	0	0	0	0	0	0
Is-54	3	0	0	1	2	0	0	0	0	0	0	0
Is-55	9	0	0	0	9	0	0	0	0	0	0	0
Is-56	2	0	0	0	2	0	0	0	0	0	0	0
Is-57	6	0	0	5	1	0	0	0	0	0	0	0
Is-58	1	0	1	0	0	0	0	0	0	0	0	0
Is-59	2	0	0	0	2	0	0	0	0	0	0	0
Is-60	2	0	0	0	2	0	0	0	0	0	0	0
Is-61	1	0	0	0	1	0	0	0	0	0	0	0
Is-62	4	0	0	0	4	0	0	0	0	0	0	0
Is-63	20	1	0	0	0	11	2	5	1	0	0	0
Is-64	1	0	0	0	1	0	0	0	0	0	0	0
Is-65	1	0	0	0	1	0	0	0	0	0	0	0
Is-66	1	0	0	0	1	0	0	0	0	0	0	0
Is-67	1	0	0	1	0	0	0	0	0	0	0	0
Is-68	1	0	0	1	0	0	0	0	0	0	0	0
Is-69	3	0	0	3	0	0	0	0	0	0	0	0
Is-70	2	0	0	2	0	0	0	0	0	0	0	0
Is-71	1	0	1	0	0	0	0	0	0	0	0	0
Is-72	2	0	0	0	0	2	0	0	0	0	0	0
Is-73	2	0	0	0	0	2	0	0	0	0	0	0
Is-74	6	0	0	0	0	6	0	0	0	0	0	0
Is-75	1	0	0	0	0	1	0	0	0	0	0	0
Is-76	3	0	0	0	0	3	0	0	0	0	0	0
Is-77	2	0	0	0	0	0	0	1	1	0	0	0
Is-78	1	0	0	0	0	1	0	0	0	0	0	0
Is-79	1	0	0	0	0	0	0	1	0	0	0	0
Total	582	2	6	96	168	166	22	100	6	3	11	2

* AB = Alberta, SK = Saskatchewan, MB = Manitoba, ON = Ontario, QC = Quebec, NB = New Brunswick, PE = Prince Edward Island, NS = Nova Scotia, and NL = Newfoundland.

A. Manitoba

Haplotype	*n*	No. individuals from:	
		PVPP	ST
Is-1	43	20	23
Is-2	6	4	2
Is-4	14	7	7
Is-6	1	1	0
Is-9	4	4	0
Is-13	1	0	1
Is-23	1	0	1
Is-30	1	1	0
Is-51	3	0	3
Is-52	2	0	2
Is-53	1	1	0
Is-54	1	1	0
Is-57	5	0	5
Is-67	1	1	0
Is-68	1	1	0
Is-69	3	3	0
Is-70	2	2	0
Total	90	46	44

B. Ontario

Haplotype	*n*	No. individuals from:	
		PPNP	LPPP
Is-1	70	28	42
Is-2	2	2	0
Is-4	1	1	0
Is-6	1	0	1
Is-7	3	0	3
Is-9	4	0	4
Is-10	2	0	2
Is-13	14	8	6
Is-15	26	2	24
Is-20	3	0	3
Is-50	3	3	0
Is-63	11	0	11
Is-72	2	2	0
Is-73	2	0	2
Is-74	6	0	6
Is-75	1	0	1
Is-76	3	0	3
Total	154	46	108

C. Minnesota

Haplotype	*n*	No. individuals from:		
		ISP	CR	CSP
Is-1	100	35	33	32
Is-2	2	0	1	1
Is-4	12	6	2	4
Is-6	5	2	0	3
Is-8	2	2	0	0
Is-9	3	2	0	1
Is-13	7	3	3	1
Is-14	3	2	0	1
Is-17	1	0	1	0
Is-23	1	0	1	0
Is-48	2	0	2	0
Is-51	1	1	0	0
Is-52	2	1	0	1
Is-53	1	1	0	0
Is-54	2	1	0	1
Is-55	9	0	5	4
Is-56	2	0	2	0
Is-57	1	0	1	0
Is-59	2	0	0	2
Is-60	2	0	2	0
Is-61	1	0	1	0
Is-62	4	0	1	3
Is-64	1	0	1	0
Is-65	1	0	0	1
Is-66	1	0	0	1
Total	168	56	56	56

D. Rhode Island

Haplotype	*n*	No. individuals from:	
		TPSK	HISK
Is-1	41	24	17
Is-2	12	6	6
Is-4	9	5	4
Is-5	3	2	1
Is-6	1	0	1
Is-7	24	8	16
Is-12	2	2	0
Is-49	1	0	1
Is-63	5	3	2
Is-77	1	0	1
Is-79	1	0	1
Total	100	50	50

Figure 2.3. The number of *I. scapularis* of the different mt 16S haplotypes collected from nine established populations in Canada (A and B) and the U.S.A. (C and D). A) PVPP = Pembina Valley Provincial Park and ST = Stanley Trail; B) PPNP = Point Pelee National Park and LPPP = Long Point Provincial Park; C) ISP = Itasca State Park, CR = Camp Ripley, and CSP = St. Croix State Park; and, D) TPSK = Trustom Pond and HISK = Hazard Island.

Table 2.6. Haplotype diversity (h) and nucleotide diversity (π) estimates, and tests of neutrality within the mt 16S rRNA gene for established populations of *I. scapularis*.

Site[a]	n	No. polymorphic sites	h	π	Tajima's test:		Fu's test:		Chakraborty's test:		
					Tajima's D	p^b	*Fs*	p^b	No. haplotypes: Exp.	Obs.	p^b
PVPP	46	12	0.7807	0.002922	-1.70183	0.02600	-7.37352	0.00000	8.49548	12	0.05268
ST	44	7	0.6945	0.002169	-1.03543	0.16100	-3.77820	0.01600	6.20740	8	0.12169
ISP	56	10	0.5994	0.001911	-1.74725	0.01900	-8.27156	0.00000	4.97816	11	0.00242
CR	56	15	0.6461	0.002396	-2.07512	0.00400	-11.57568	0.00000	5.69401	14	0.00018
CSP	56	13	0.6656	0.002126	-1.91121	0.00400	-12.74976	0.00000	6.03913	14	0.00038
PPNP	46	6	0.6019	0.002064	-1.29154	0.08700	-2.73859	0.04900	4.79316	7	0.09335
LPPP	108	13	0.7856	0.003176	-1.28403	0.08100	-5.53207	0.02400	11.03984	13	0.10475
TPSK	50	7	0.7273	0.003157	-0.06102	0.48700	-1.14035	0.27600	7.16190	7	0.18523
HISK	50	8	0.7731	0.003571	-0.08520	0.52600	-3.41495	0.04700	8.46104	10	0.12896

[a] Ticks from MB: PVPP = Pembina Valley Provincial Park and ST = Stanley Trail; MN: ISP = Itasca State Park, CR = Camp Ripley, and CSP = St. Croix State Park; ON: PPNP = Point Pelee National Park and LPPP = Long Point Provincial Park; and South Kingstown, RI: TPSK = Trustom Pond and HISK = Hazard Island.

[b] Significance levels are $p < 0.02$ for Fu's test and $p < 0.05$ for Tajima's and Chakraborty's tests.

Although the observed numbers of haplotypes in six populations (7-14; Table 2.6 and Fig. 2.3) were not significantly different from the expected number based on Chakraborty's test, significantly more haplotypes were detected than expected from the three populations in MN (Table 2.6). Tajima's test for neutrality indicated that there were significant negative departures from zero for four tick populations (PVPP, ISP, CR, and CSP) (Table 2.6). Similarly, the F_S statistics of Fu revealed that the F_S values for five populations (all those sampled from MB and MN) were significantly different from zero (Table 2.6). Comparison of the F_{ST} values among the established tick populations showed that there were significant differences between most pairs of populations (Table 2.7). The exceptions to this were between ISP (MN) and the three populations in PVPP (MB), ST (MB), and CSP (MN); between PVPP (MB) and ST (MB); and between CR (MN) and CSP (MN). The Mantel Test (Fig. 2.4), which compared pair-wise F_{ST} values as a factor of geographical distance between tick populations showed that there was a positive and significant association between genetic and geographical distances ($b = 0.000058$, $r^2 = 0.299$, $p = 0.002$).

The combined rarefaction and extrapolation curves as well as those representing the estimated total haplotype richness (Chao 1) for ST, ISP, CR, PPNP, and LPPP converged on an asymptote, while those for PVPP, CSP, and HISK did not (Fig. 2.5). For TPSK, the rarefaction curve as well as the curve of the non-parametric estimator Chao 1 also converged on an asymptote. The rarefaction and extrapolation curves in addition to the curves of the non-parametric estimator Chao 2 for the "eastern" (i.e., PPNP, LPPP, TPSK, and HISK) and "western" (i.e., ST, PVPP, ISP, CR, and CSP) established populations of *I. scapularis* failed to converge on an asymptote, and they suggested that the estimated haplotype richness in the west was greater than in the east (Fig. 2.6). However, since the 95% confidence intervals of the curves for the "eastern" and "western" regions overlapped, there was no significant difference in the estimated number of haplotypes between the tick populations in these two regions. For the nine established populations of *I. scapularis*, the rarefaction and extrapolation curves did not reach an asymptote (Fig. 2.7); however, the curve of the Chao 2 estimator did, indicating that the number of haplotypes to be expected upon exhaustively sampling *I. scapularis* from the different localities in this study was ~82. The scatter plot of the number of haplotypes detected in each of the nine populations against the total number of ticks collected from each population on the rarefaction curve suggested that more haplotypes were detected in the west (i.e., PVPP, ST, ISP,

Table 2.7. Pair-wise comparisons of geographical (km; upper diagonal) and genetic (F_{ST} values; lower diagonal) distances among established populations of *I. scapularis*.

	PVPP[a]	ST	ISP	CR	CSP	PPNP	LPPP	TPSK	HISK
PVPP	--	29	307	426	543	1452	1554	2241	2246
ST	0.01872	--	326	447	560	1465	1565	2247	2252
ISP	0.01136	0.01095	--	123	238	1157	1272	1978	1983
CR	0.04177***	0.02911***	0.02143*	--	145	1061	1186	1904	1909
CSP	0.02402*	0.02309*	0.00315	0.00332	--	921	1042	1758	1763
PPNP	0.04741**	0.04162***	0.02194*	0.02249**	0.03432***	--	188	909	914
LPPP	0.06923***	0.07429***	0.06040***	0.04590***	0.06036***	0.04617***	--	738	743
TPSK	0.04613***	0.07204***	0.08473***	0.06644***	0.08303***	0.07878***	0.07007***	--	5
HISK	0.18732***	0.23045***	0.25897***	0.21602***	0.24510***	0.23932***	0.20120***	0.05159***	--

*** Significance level $p < 0.001$.
** Significance level $p < 0.01$.
* Significance level $p < 0.05$.
[a] MB: PVPP = Pembina Valley Provincial Park and ST = Stanley Trail; MN: ISP = Itasca State Park, CR = Camp Ripley, and CSP = St. Croix State Park; ON: PPNP = Point Pelee National Park and LPPP = Long Point Provincial Park; and South Kingstown, RI: TPSK = Trustom Pond and HISK = Hazard Island.

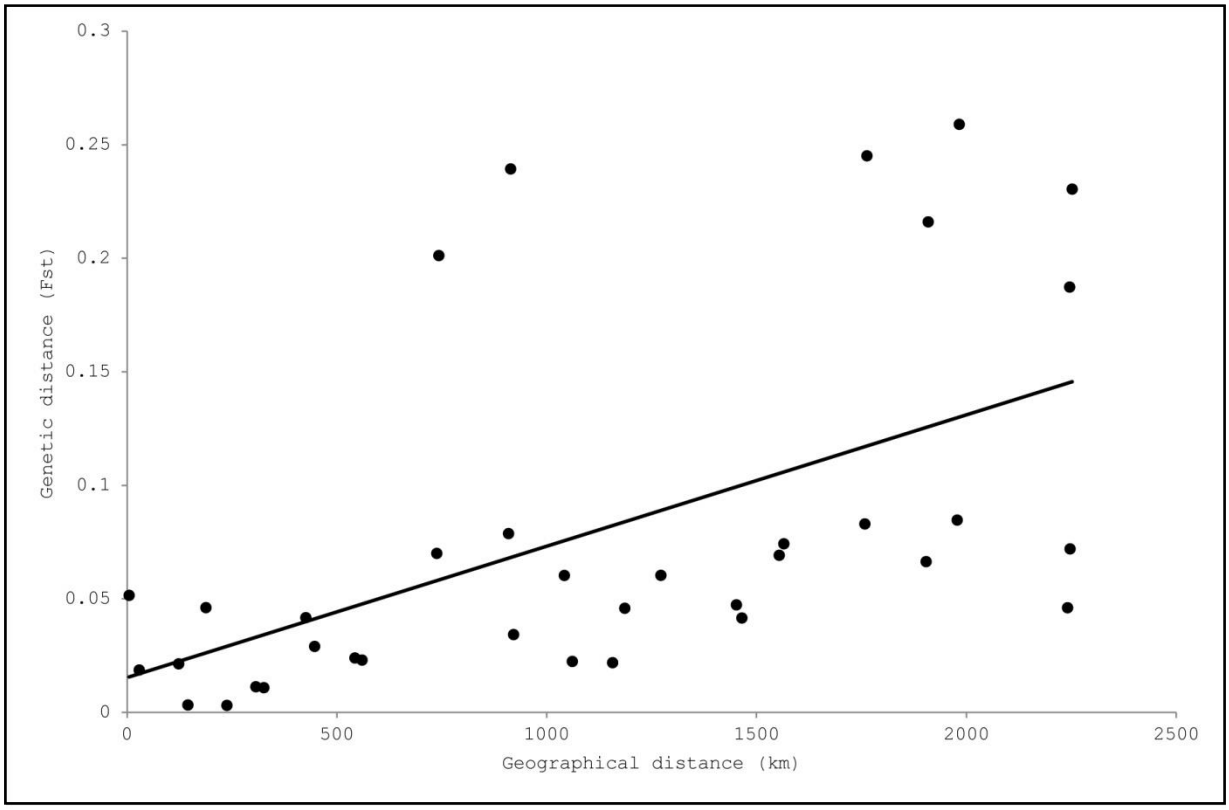
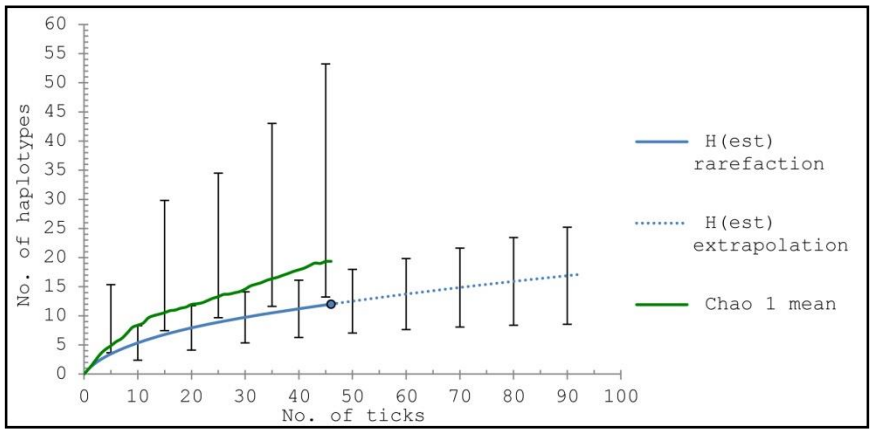
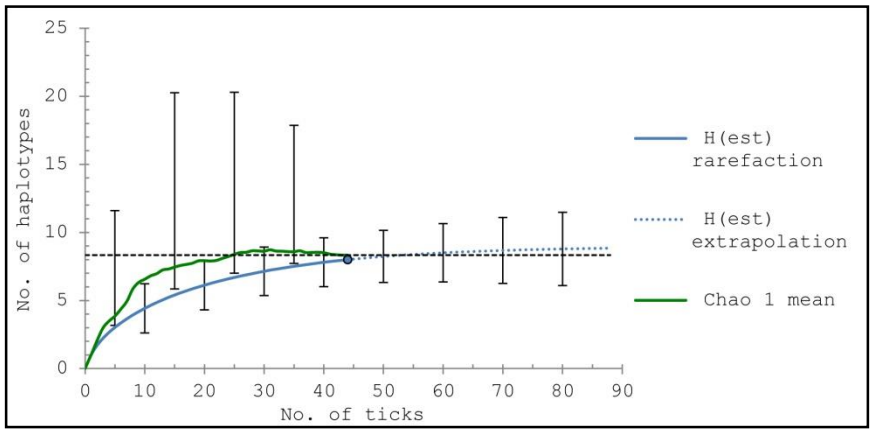


Figure 2.4. Scatter plot depicting the pair-wise comparisons of genetic (F_{ST}) and geographical (km) distances among the nine established populations of *I*. *scapularis* in Canada and the U.S.A.

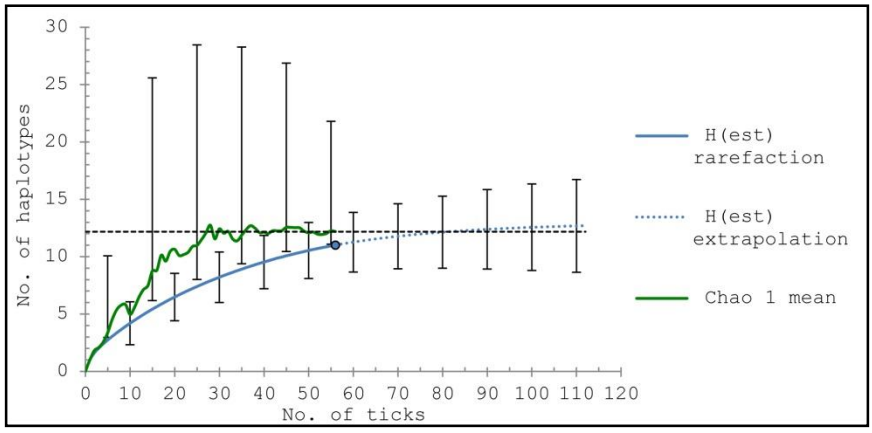
A. PVPP



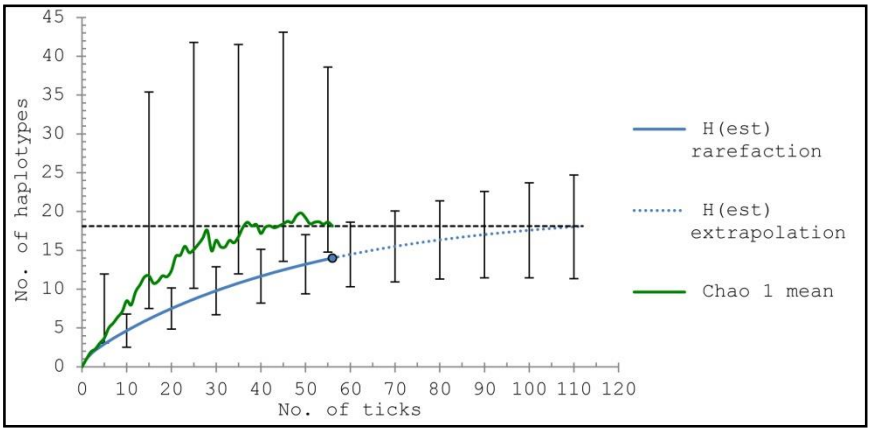
B. ST



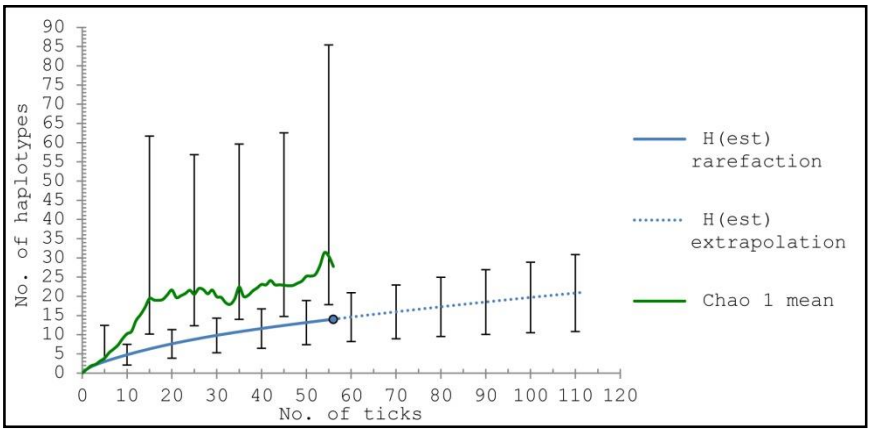
C. ISP



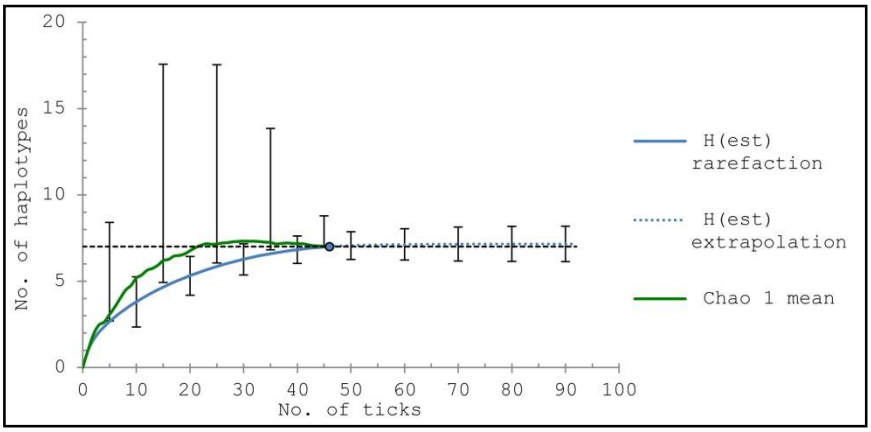
D. CR



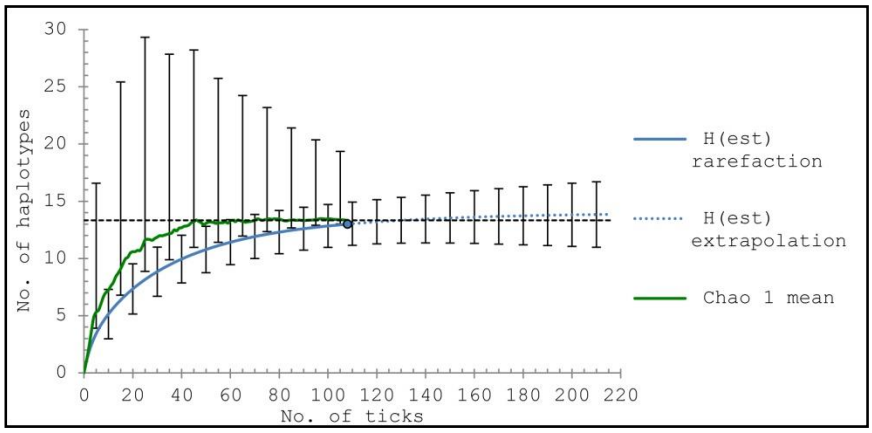
E. CSP



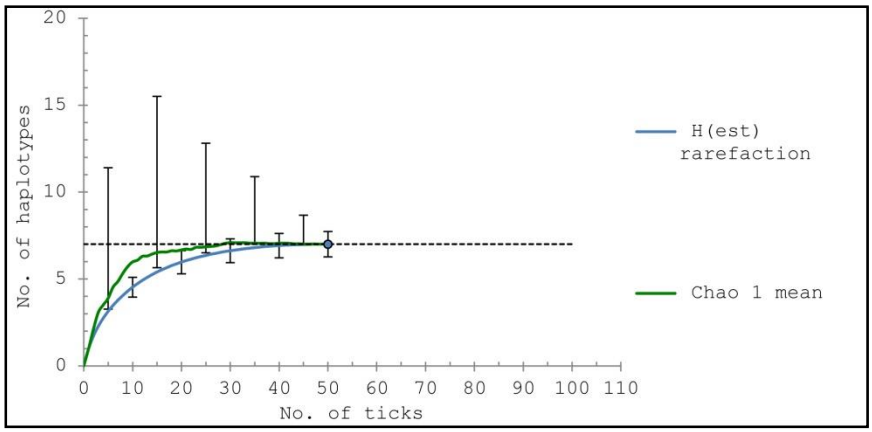
F. PPNP



G. LPPP



H. TPSK



I. HISK

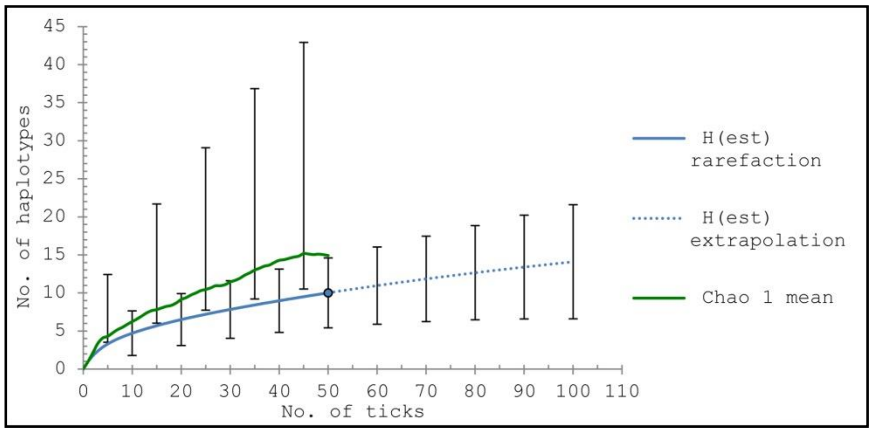


Figure 2.5. Rarefaction, extrapolation, and estimated total haplotype richness (Chao 1) curves with 95% confidence intervals for each of the nine established populations of *I. scapularis* in Canada and the U.S.A.: A) Pembina Valley Provincial Park, B) Stanley Trail, C) Itasca State Park, D) Camp Ripley, E) St. Croix State Park, F) Point Pelee National Park, G) Long Point Provincial Park, H) Trustom Pond, South Kingstown, and I) Hazard Island, South Kingstown. Asymptotes are denoted by black dashed lines.

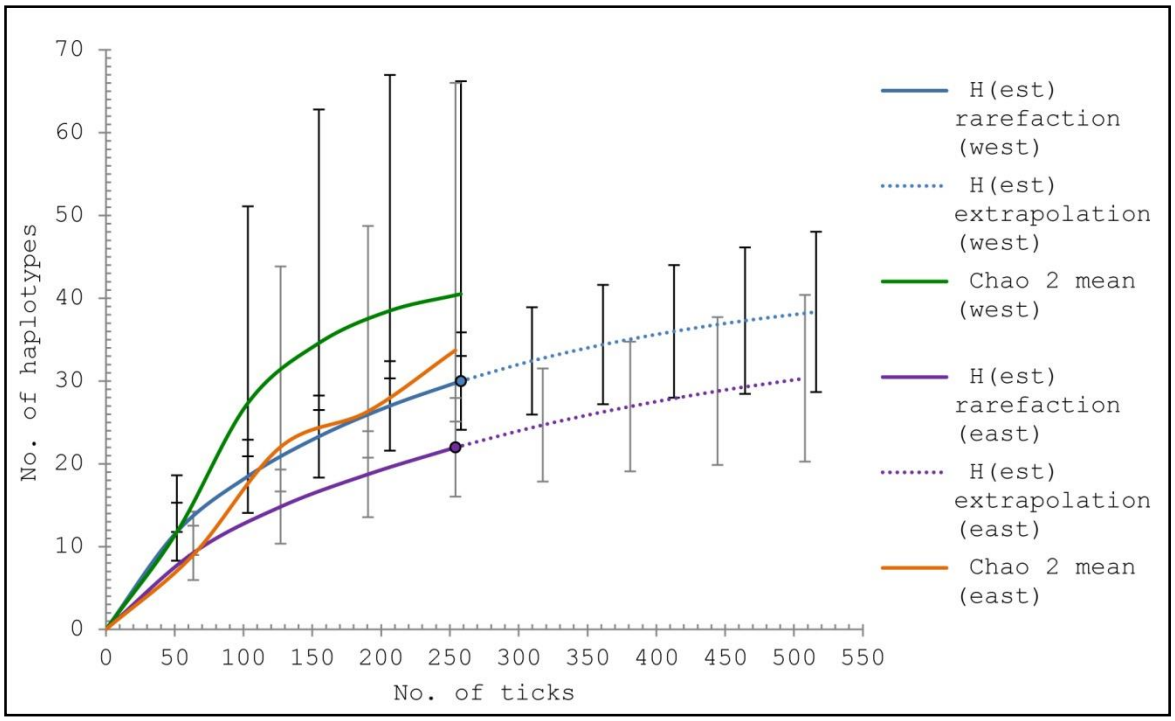


Figure 2.6. Rarefaction, extrapolation, and estimated total haplotype richness (Chao 2) curves with 95% confidence intervals for the five established populations of *I. scapularis* in the "western" (Pembina Valley Provincial Park, Stanley Trail, Itasca State Park, Camp Ripley, and St. Croix State Park) and for the four in the "eastern" (Point Pelee National Park, Long Point Provincial Park, Trustom Pond, South Kingstown, and Hazard Island, South Kingstown) areas of the U.S.A. and Canada.

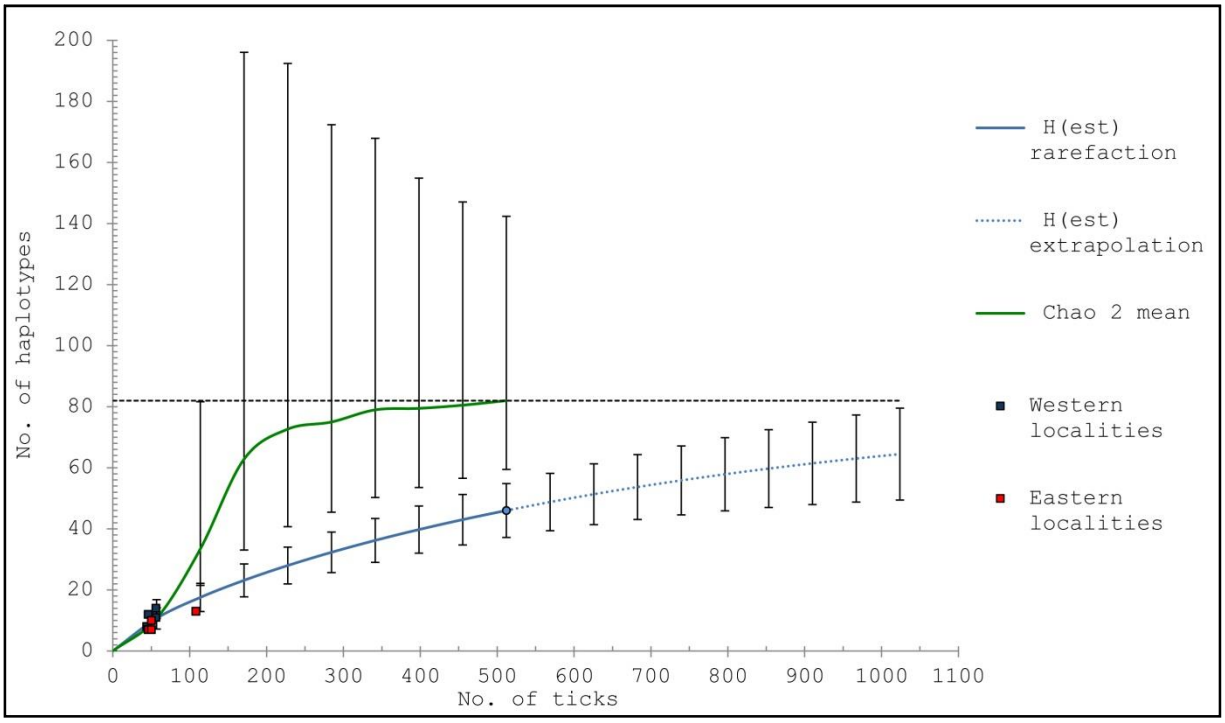


Figure 2.7. Rarefaction, extrapolation, and estimated total haplotype richness (Chao 2) curves with 95% confidence intervals for the nine established populations of *I. scapularis* in the U.S.A. and Canada, and scatter plot of the number of haplotypes found at each population against the total number of ticks collected from each (see Fig. 2.3 for data). The asymptote of the estimated total haplotype richness curve is denoted by the black dashed line.

CR, and CSP) for a given sample size than would be expected (i.e., each point laid on or above the curve), while fewer haplotypes were detected in the east (i.e., PPNP, LPPP, TPSK, and HISK) for a given sample size than would be expected (i.e., each point laid on or below the curve). The point corresponding to LPPP fell below the 95% confidence intervals of the "eastern" curves. However, there was no significant difference in the estimated number of haplotypes between the tick populations in the west and east given the overlap in 95% confidence intervals of the curves.

The AMOVA test (Table 2.8) indicated strong genetic structure among populations both within and among different geographical regions. Most of the variance (94.3%) occurred within populations. Nonetheless, there are many shared haplotypes among populations in different geographical regions (Fig. 2.3).

The tree produced from the phylogenetic analyses of the sequence data is shown in Fig. 2.8. The bootstrap analysis supported the separation of the 16S *I. scapularis* haplotypes into the "southern" and "American" clades. There was little statistical support for the different branches in the "American" clade.

Fig. 2.9A depicts the network relationships among 74 haplotypes (see Table 2.4) belonging to the two major clades: the "southern" and "American" clades, which differed from one another by more than 11 bp. The 52 haplotypes detected in this study (see Tables 2.3, 2.4, and 2.5) belonged to the "American" clade. The minimum spanning network tree of this group (Fig. 2.9B) exhibited a "star" pattern where haplotype Is-1 represented the central haplotype from which the other haplotypes of the clade differed by 1-6 bp. The number of base pair differences between pairs of haplotypes of the "American" clade ranged from 1-9 bp (Fig. 2.9B). The next six most frequently detected haplotypes of this study (i.e., haplotypes Is-2, Is-4, Is-7, Is-13, Is-15, and Is-63) each differed by one mutational change from the central haplotype, except for haplotype Is-7 which differed from haplotype Is-1 by 2 bp through haplotype Is-2 (Fig. 2.9B). These common haplotypes, apart from haplotype Is-7, each represented a link (i.e., secondary node) from haplotype Is-1 to between two and 12 haplotypes (Fig. 2.9B). Haplotype Is-7 represented a tertiary node from the central haplotype to seven haplotypes (Fig. 2.9B). In contrast, haplotypes belonging to the "southern" clade did not have a central haplotype and differed from one another by 1-11 bp (Fig. 2.9A).

Table 2.8. Analysis of Molecular Variance (AMOVA) for nine established populations of *I. scapularis* from Canada and the U.S.A.

Variance component	*df*	% variance	Fixation index	*p*
Among regions[a]	3	3.8	Φ_{CT} = 0.03791	< 0.0001
Among populations within regions	5	1.9	Φ_{SC} = 0.01947	< 0.005
Within populations	503	94.3	Φ_{ST} = 0.05665	< 0.0001

[a] Regions = Manitoba (PVPP, ST), Ontario (PPNP, LPPP), Minnesota (ISP, CR, CSP) and Rhode Island (TPSK, HISK).

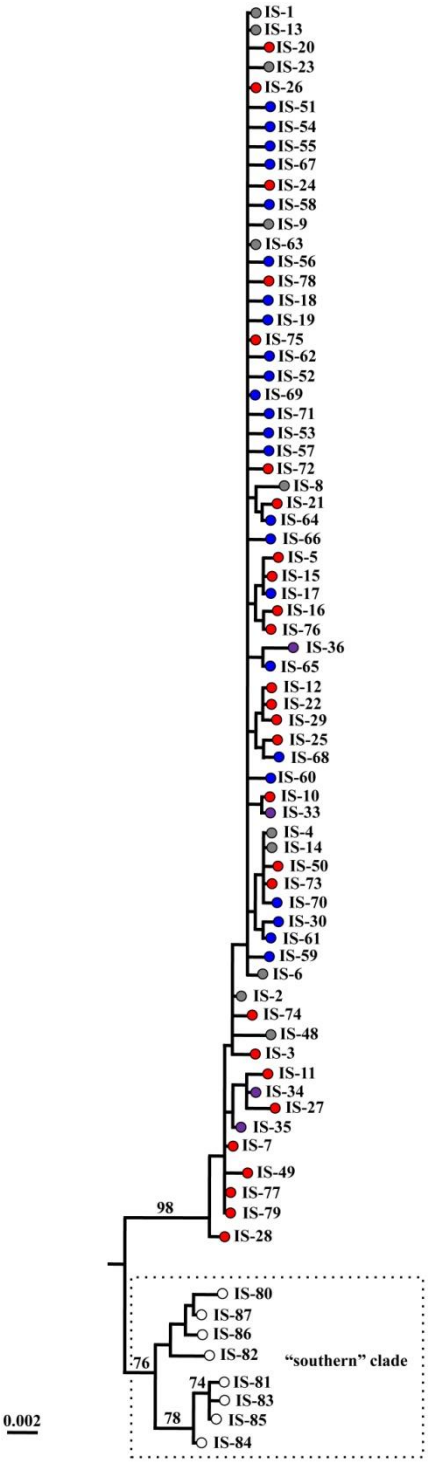


Figure 2.8. Neighbour-joining (NJ) tree depicting the relationships among the 74 mt 16S rRNA gene haplotypes of *I. scapularis* detected in this and other studies (Caporale et al. 1995; Rich et al. 1995; Norris et al. 1996; Qiu et al. 2002; Anstead and Chilton 2011; Krakowetz et al. 2011). The numerical designations of Krakowetz et al. (2011) were expanded upon as different systems have been used for haplotype designations in other studies (see Table 2.4). The "southern" (black rectangle) and "American" clades are shown. Haplotypes belonging to the "American" clade are denoted by purple (Norris et al. 1996 only), or blue, grey, or red circles depending on whether each was detected in the "western," "western" and "eastern," or "eastern" geographical areas, respectively. A DNA sequence of *I. pacificus* was used to root the tree. Numbers above the branches indicate bootstrap values (>70%) for the NJ analyses.

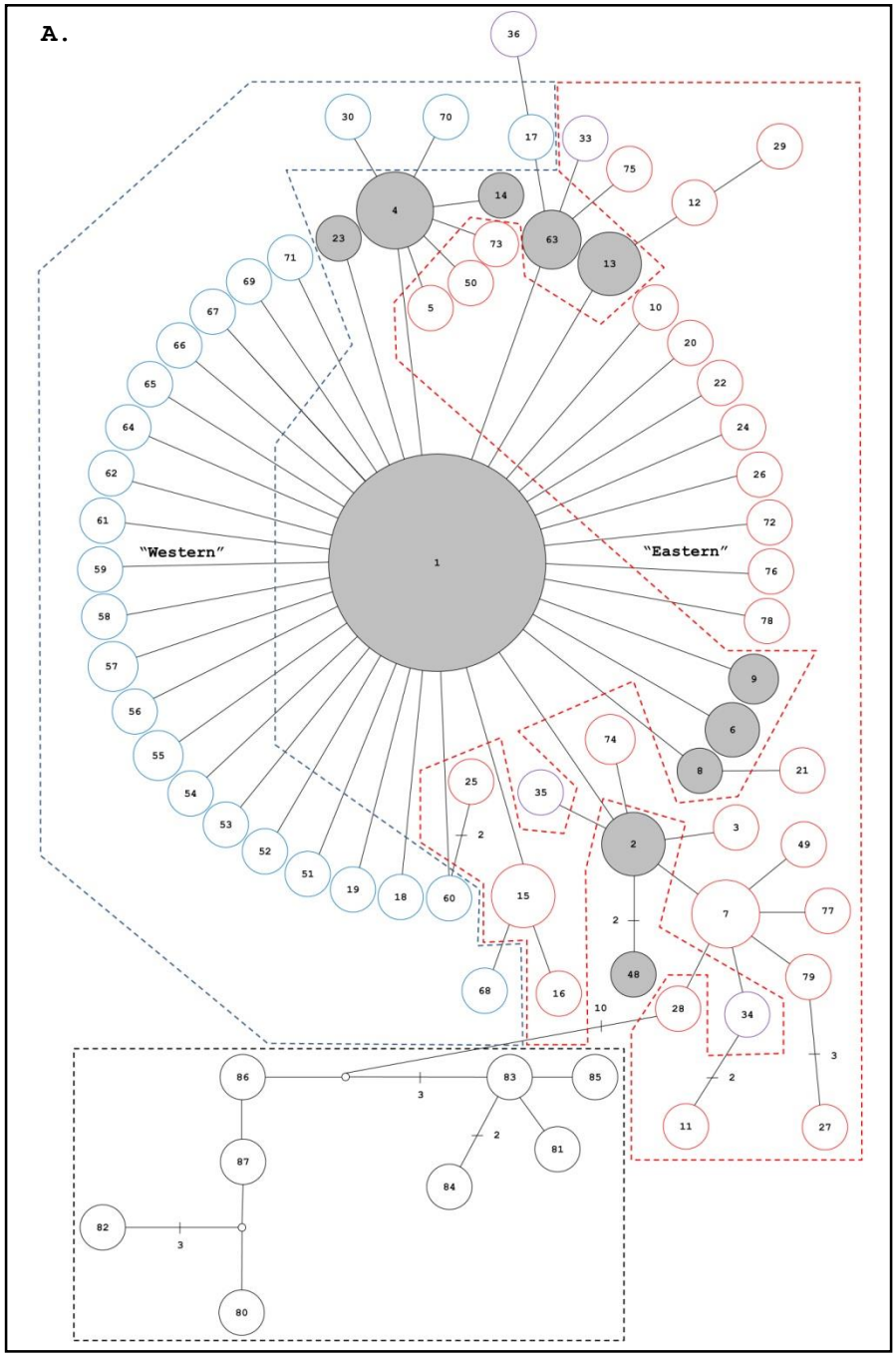


Figure 2.9. A minimum spanning network tree depicting the relationships among the mt 16S rRNA gene haplotypes of *I. scapularis* detected in this and other studies (Caporale et al. 1995; Rich et al. 1995; Norris et al. 1996; Qiu et al. 2002; Anstead and Chilton 2011; Krakowetz et al. 2011). The numerical designations of Krakowetz et al. (2011) were expanded upon as different systems have been used for haplotype designations in other studies (see Table 2.4). The number of nucleotide differences between pairs of DNA sequences (circles) is one unless otherwise indicated. The size of each circle is proportional to the number of ticks of that haplotype (this study only). Putative haplotypes are represented by small open circles. A) Depicts the "southern" (black rectangle and circles) and "American" (blue, grey, red, and purple circles) clades. B) Depicts the "American" clade only.

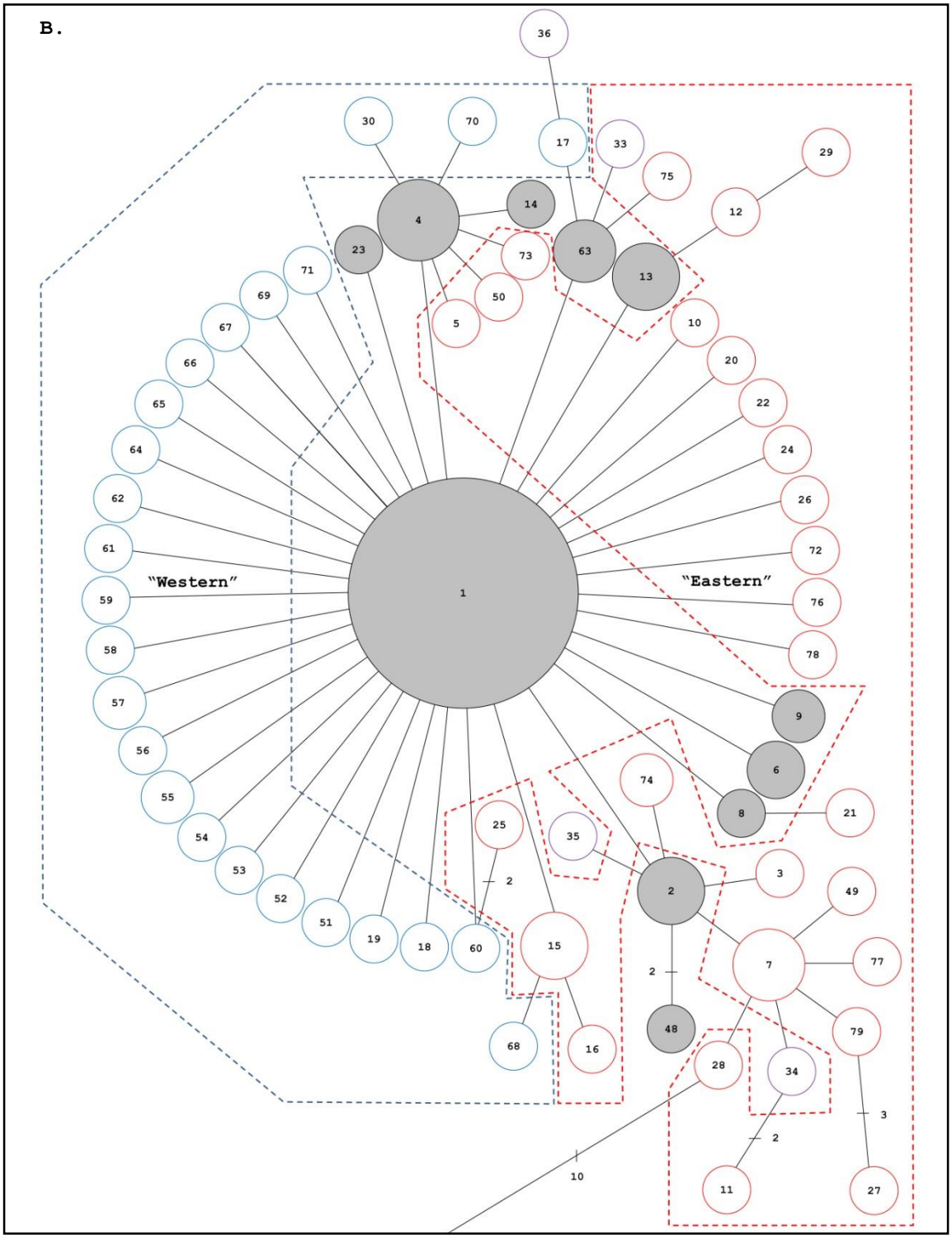


Figure 2.9. Continued. Haplotypes belonging to the "American" clade are denoted by purple (Norris et al. 1996 only), or blue, grey, or red circles depending on whether each was detected in the "western," "western" and "eastern," or "eastern" geographical areas, respectively. Haplotypes only associated with "western" geographical regions of Canada and the U.S.A. are contained within the blue polygon, while those only associated with "eastern" regions are delimited by the red one.

Blacklegged ticks representing 11 of the mt 16S rRNA gene haplotypes of the "American" clade (i.e., haplotypes Is-1, Is-2, Is-4, Is-6, Is-8, Is-9, Is-13, Is-14, Is-23, Is-48 and Is-63) have been collected in both the "eastern" and "western" geographical areas (Fig. 2.9B). For the purposes of this study, the "eastern" region included *I. scapularis* collected in the Atlantic and Central Provinces of Canada (NL, NS, Prince Edward Island (PE), NB; and QC and ON, respectively), and in RI, U.S.A., whereas the "western" region included ticks collected in the Prairie Provinces of Canada (AB, SK and MB) and in MN, U.S.A. Additionally, the "eastern" region included *I. scapularis* collected from the provinces of ON and NS (Krakowetz et al. 2011); and the U.S.A. states of NY, MA, RI, CT, New Jersey, PA, and Maryland (MD) (Qiu et al. 2002), and NY, MA, and MD (Rich et al. 1995), while the "western" region included ticks from MB (Krakowetz et al. 2011) and SK (Anstead and Chilton 2011), Canada; and Illinois and WI, U.S.A. (Rich et al. 1995) (Fig. 2.9B). In contrast, blacklegged ticks representing 51 haplotypes of the "American" clade have, thus far, been collected from either the "eastern" or "western" geographical regions (i.e., 27 and 24 haplotypes, respectively) (Fig. 2.9B). Of the remaining haplotypes of this clade, ten that differed from the central haplotype by 1-2 bp were associated with ticks that were collected from both geographical areas. Of these, half (i.e., five haplotypes; haplotypes Is-2, Is-4, Is-8, Is-13 and Is-63) represented secondary nodes from which several haplotypes (i.e., 25 haplotypes) arose. Four (i.e., haplotypes Is-2, Is-8, Is-13, and Is-63) of the 10 haplotypes associated with both the "eastern" and "western" geographical areas represented a link from haplotype Is-1 to several haplotypes (i.e., 19 haplotypes) that were associated with the "eastern" geographical area only. In contrast, none of these 10 haplotypes represented a link from Is-1 to haplotypes that were exclusive to the "western" geographical area. The remaining five of these 10 haplotypes comprised either a secondary node (i.e., haplotype Is-4) from which haplotypes associated with the "eastern" and "western" geographical areas arose or terminal secondary (i.e., haplotypes Is-6 and Is-9) or tertiary (i.e., haplotypes Is-14 and Is-48) nodes. Remarkably, one haplotype that has been detected in the "western" geographical area only (i.e., haplotypes Is-60) represented a link to an "eastern" haplotype (i.e., haplotype Is-25), whereas one haplotype that has been detected in the "eastern" geographical area only (i.e., haplotype Is-15) represented a secondary node from which one "eastern" and one "western" haplotype arose.

Fig. 2.10 shows which haplotypes from this study occur in each of four, broad geographical areas (i.e., "western" Canada, "eastern" Canada, "western" U.S.A., and "eastern" U.S.A.). A greater number of haplotypes (i.e., 33 haplotypes; $n = 272$) were detected in the "western" areas of Canada and U.S.A. than in the "eastern" areas (i.e., 26 haplotypes; $n = 310$). The number of haplotypes in common between "western" U.S.A. and "western" Canada (i.e., 13 haplotypes) was approximately twice as great as the number in common between the "eastern" U.S.A. and "eastern" Canada (i.e., seven haplotypes). Ticks collected from Canada represented 36 haplotypes ($n = 314$), while those from the U.S.A. represented 32 ($n = 268$). The number of haplotypes in common between the "western" and "eastern" geographical areas of Canada (i.e., seven haplotypes) was nearly twice as great as the number in common between the "western" and "eastern" areas of the U.S.A (i.e., four haplotypes). Among the four geographical areas, the number of haplotypes common to all areas was four.

Fig. 2.10 also shows that the number of haplotypes that were unique to "eastern" Canada was 13, which was approximately twice as great as the number that were unique to "western" Canada (i.e., seven haplotypes). In contrast, the number of haplotypes that were associated with ticks from the "western" U.S.A. only (i.e., 12 haplotypes) was thrice the number that were unique to the "eastern" U.S.A (i.e., four haplotypes). By controlling for the differing sample sizes among the four regions, the sampling effort (i.e., the number of ticks) necessary to detect a single additional haplotype in "western" Canada and "western" U.S.A. was 5 ticks (e.g., $n = 104$ / 21 haplotypes) and 7 ticks, respectively. In contrast, the numbers of ticks required to detect an additional haplotype in "eastern" Canada and "eastern" U.S.A. were 10 and 9 ticks, respectively. Similarly, the sampling effort necessary to detect an additional haplotype that would be unique to a geographical area was 15 ticks (e.g., $n = 104$ / 7 unique haplotypes; and $n = 168$ / 12 unique haplotypes) in "western" Canada and "western" U.S.A., respectively, and 16 and 25 ticks in "eastern" Canada and "eastern" U.S.A., respectively.

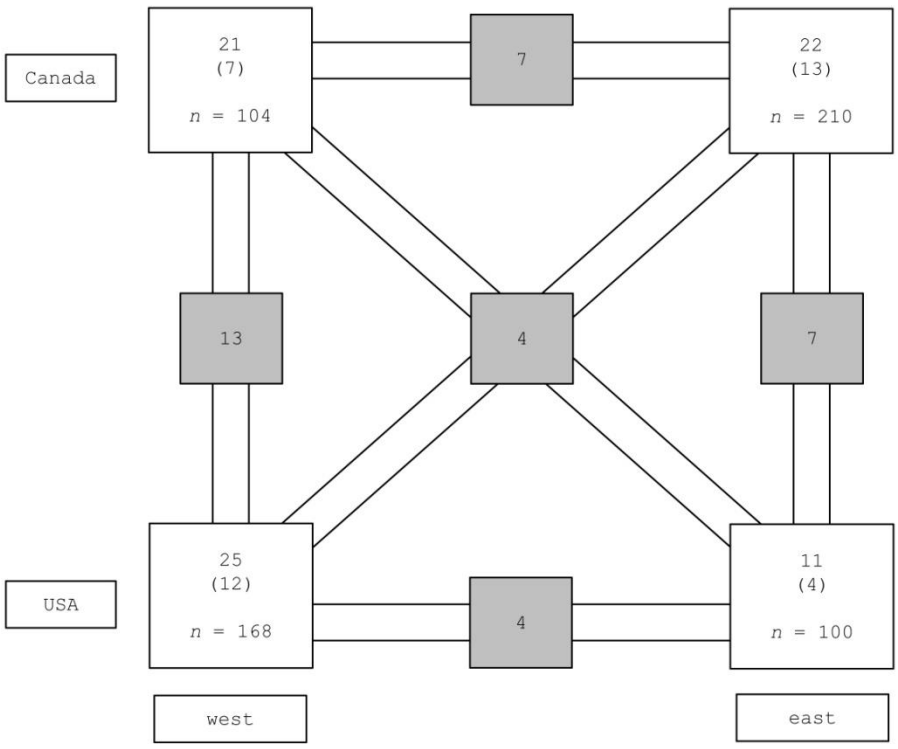


Figure 2.10. Diagram representing the number of mt 16S rRNA gene haplotypes found in key geographical areas (i.e., "western" Canada, "eastern" Canada, "western" U.S.A., and "eastern" U.S.A.). The white boxes show the number of haplotypes found in a particular region and those exclusive to a region (in parentheses) in addition to the corresponding sample size (*n*). The grey boxes describe the number of haplotypes detected in all (center) and each pair (periphery) of regions. Haplotypes of the ticks collected from the nine established populations (*n* = 512) and those of the adventitious ticks (*n* = 70) have been included.

2.5. Discussion

2.5.1. Genetic diversity

A total of 52 haplotypes of the mt 16S rRNA gene that belonged to the "American" clade, as defined by Qiu et al. (2002), were detected among the 582 *I. scapularis* collected from southern Canada, and Minnesota and Rhode Island (U.S.A.). Thirty (i.e., 58%) of these haplotypes are "new" in that they have not been reported previously in other studies of *I. scapularis* conducted in the Northeast or Upper Midwest of the U.S.A. (Caporale et al. 1995, Rich et al. 1995, Norris et al. 1996, Qiu et al. 2002) or in southern Canada (Anstead and Chilton 2011, Krakowetz et al. 2011). In the present study, the number of mt 16S rRNA gene haplotypes of *I. scapularis* detected was much higher than that reported in similar studies (i.e., 7-29 haplotypes) (Rich et al. 1995, Norris et al. 1996, Qiu et al. 2002, Trout et al. 2009, Krakowetz et al. 2011, Van Zee et al. 2013), even when comparisons are made from the same geographical areas. For example, 11 haplotypes were detected among 100 questing *I. scapularis* nymphs from Trustom Pond and Hazard Island in Washington County (RI) (this study), whereas only four haplotypes had been reported previously among 27 *I. scapularis* adults collected in Washington County (Qiu et al. 2002). All four of these haplotypes in the study by Qiu et al. (2002) were detected among ticks collected from Rhode Island in the present study. Similarly, 14 haplotypes were detected in 56 *I. scapularis* adults from Camp Ripley in Morrison County (MN) (this study), while only three haplotypes were detected previously among 19 ticks from Morrison County (Norris et al. 1996). These results suggest that sample size is an important determinant of the number of haplotypes detected within a tick population. This is further supported by comparing the number of haplotypes among *I. scapularis* collected from the same established tick populations in southern Canada in the present study to that reported in a previous study by Krakowetz et al. (2011). For example, Krakowetz et al. (2011) detected five haplotypes among 26 adult ticks collected from PPNP in southern Ontario, while all but one haplotype, and three additional haplotypes (i.e., a total of seven haplotypes), were detected in a second sample of 46 ticks from this population in the present study. The effect of sample size on estimates of haplotype number is also evident by comparisons of ticks collected from the established tick population in LPPP in southern Ontario. Krakowetz et al. (2011) detected six haplotypes among 26 adult ticks from LPPP, whereas all but one of these haplotypes, and an additional eight

haplotypes (i.e., a total of 13 haplotypes), were detected among 108 adult ticks in the present study.

The number of mt 16S rRNA gene haplotypes detected within established populations of *I. scapularis* in the present study ranged from 7-14, which was significantly greater than the number of haplotypes for this gene reported for other species of *Ixodes* in North America. For example, only a small number (i.e., 1-3) of haplotypes were detected for *Ixodes angustus*, *Ixodes kingi* and *Ixodes sculptus*, despite large sample sizes (i.e., 271, 88, and 55 individuals, respectively) (Anstead and Chilton 2011, Anstead et al. 2013, Anstead et al. 2014). Explanations for differences in haplotype number among different taxa may be associated with differences in evolutionary mechanisms (e.g., hitchhiking effects, AT content, or repair efficiency; Schmitz and Moritz 1998); however, because all four tick species are closely related, this is unlikely to account for the differences observed. Ecological (e.g., habitat, seasonal activity, host-seeking behaviour, host-usage, and life span) and biological (e.g., number of nymphal instars, feeding habits, weight gain while feeding, and osmoregulation) differences among the taxa (Parola and Raoult 2001) are the more likely explanations for the difference in haplotype number between *I. scapularis* and the other three species of *Ixodes*. For example, host preference has been shown to have influenced the phylogeography of two African ixodid tick species (Cangi et al. 2013) and to have influenced the coevolutionary relationships between parasitic protozoa of the genus *Leucocytozoon* and their avian hosts (Jenkins et al. 2012). It has been suggested that host traits, such as migratory behaviour, can facilitate the dispersal of economically-important parasites and impact the diversity of the parasites harbored by these migratory hosts (Jenkins et al. 2012). Therefore, the greater numbers of mt 16S rRNA gene haplotypes that have been reported for *I. scapularis* (Rich et al. 1995, Norris et al. 1996, Qiu et al. 2002, Trout et al. 2009, Krakowetz et al. 2011, Van Zee et al. 2013), as compared to other species of *Ixodes* (see Anstead and Chilton 2011, Anstead et al. 2013, Anstead et al. 2014) in North America may be explained by biological or ecological differences (e.g., host behaviour) among them. For example, *I. angustus*, *I. kingi*, and *I. sculptus* primarily parasitize small mammals (e.g., non-migrant hosts such as wood rats, squirrels, mice, shrews, badgers, dogs, ground squirrels, and pocket gophers; Bishopp and Trembley 1945, Anstead and Chilton 2011, Anstead et al. 2013, Anstead et al. 2014), which limits the dispersal of ticks to relatively small distances (e.g., ~400 m/yr based on the estimated dispersal distances for two species of pocket gophers; Hafner et al. 1998). In contrast, larval and

nymphal *I. scapularis* parasitize migratory birds, which are known to carry these ticks over large distances (i.e., up to 425 km over 5 days if an immature feeds to repletion; Ogden et al. 2005).

In the present study, 15 of the 52 haplotypes were each represented by a single tick, suggesting that there may be more haplotypes of *I. scapularis* within the geographical regions sampled from that remain undetected. This inference is supported by the rarefaction, extrapolation, and estimated total haplotype richness (Chao 2) curves, which show that the observed number of haplotypes (i.e., 52) is an underestimate of the total number of haplotypes expected (i.e., 82). Similar curves for each of the nine established populations indicate that the numbers of haplotypes detected in five populations (i.e., PVPP, ISP, CR, CSP, and HISK) are fewer than expected. An extrapolation curve for the population in TPSK could not be constructed due to the observed and expected numbers of haplotypes being equal, indicating that the total number of haplotypes detected from ticks belonging to this population represented a reliable estimate of the total haplotype richness there. According to Colwell and Coddington (1994), the observed and expected number of haplotypes can be considered equal when every haplotype occurs at least twice. Chakraborty's test revealed that the total number of mt 16S rRNA gene haplotypes detected in three of the nine established populations of *I. scapularis* (i.e., ISP, CR, and CSP, MN) was greater than the expected number of haplotypes, indicating that an excess number of rare haplotypes was observed in each of these populations (Chakraborty 1990).

The minimum spanning network analysis revealed that the most common haplotype in all established populations of *I. scapularis* examined in southern Canada, and Minnesota and Rhode Island in the U.S.A. (i.e., haplotype Is-1), also represented the central haplotype of the network. Most of the other haplotypes, which differed in DNA sequence from haplotype Is-1 by 1 bp, formed a star-shaped pattern around the central haplotype. There were another seven haplotypes, which differed from haplotype Is-1 by a single mutational change, that represented secondary nodes from which several (i.e., 19) haplotypes differed in DNA sequence by 1-2 bp. This suggests that many of the mt 16S rRNA gene haplotypes have been derived from either the central haplotype or from one of the secondary haplotypes, as a consequence of a single mutational change (i.e., a transition, transversion, or indel) in the DNA sequence of the mt 16S rRNA gene.

The mutational differences in the DNA sequences of the 52 haplotypes detected in the present study were examined in relation to a secondary structure model of the mt 16S rRNA (i.e.,

Domains IV and V) that was constructed for the central haplotype. The DNA sequences of the other haplotypes were aligned with respect to the primary sequence and secondary structure of haplotype Is-1. Mutational changes were detected at 42 of the 408 positions in the aligned DNA sequences of the 52 haplotypes. There were very few indels (i.e., four), and multiple mutational changes occurred at only one variable position. There were twice as many transitional changes (i.e., 25) as compared to the number of transversional changes (i.e., 12). This 2:1 ratio of transitional to transversional mutations differs from that reported for *I. scapularis* by Norris et al. (1996), where there was an approximately 1:1 ratio; however, the calculations of this earlier study also included haplotypes of the "southern" clade. The greater number of transitional to transversional changes could possibly be explained by tautomeric shifts (i.e., a mechanism by which transitional changes arise) (Brown et al. 1968, Spengler and Singer 1981, Singer and Spengler 1982, Griffiths et al. 2000). There was no evidence of directional mutational changes (e.g., G→A versus A→G). A large proportion (i.e., 61%) of the mutational changes in DNA sequence among haplotypes occurred at unpaired sites in the secondary structure, and, hence, did not influence base pairing in the different helices. However, 26% of the mutational changes in DNA sequence represented a partial-compensatory base pair change that maintained the secondary structure of the mt 16S rRNA. Partial-compensatory base pair changes in the paired regions of the secondary structures of rRNAs are common and indicate that many mutations at the different sequence positions of these genes are not occurring independently of one another (Higgs 1998, Li et al. 2008). In addition, the base pair differences in DNA sequence among *I. scapularis* haplotypes were not evenly distributed across Domains IV and V near the 3' terminal region of the mt 16S rRNA gene. Mutational changes at 25 (i.e., 60%) of the 42 variable positions in the aligned DNA sequences occurred within a region of the mt 16S rRNA gene that comprised only 126 bp (i.e., 31%) of the 408 bp examined. This region of the mt 16S rRNA gene, sometimes referred to as the "hypervariable region" (e.g., Smith and Bond 2003), has been shown to be highly variable in DNA sequence both within and among closely-related species of a diverse range of organisms (e.g., Gutell and Fox 1988, Gutell et al. 1993, Gutell 1996). The remaining 17 (i.e., 40%) of the variable positions in the aligned mt 16S rRNA gene sequences occurred in the 5' and 3' flanking regions of the "hypervariable region," which comprised 282 (i.e., 69%) of the base pairs there. Examination of the DNA sequence data for Eurasian and north-African populations of a closely-related species, *I. ricinus*, published by Noureddine

et al. (2011), also revealed a similar pattern of unequal mutation rates in the two regions of Domains IV and V of the mt 16S rRNA gene (Fig. 2.11). In *I. ricinus*, of the 20 variable positions in the aligned sequence (i.e., alignment length of 414 bp), which was modified according to the predicted secondary structure of the rRNA using the same methodology as for *I. scapularis*, 75% occurred within the "hypervariable region" (Fig. 2.11). These results suggest that there may be fewer structural constraints on the "hypervariable region" of the mt 16S rRNA as compared to the flanking regions that may be associated with the function of the ribosome (Misof et al. 2002).

2.5.2. Population genetic structure

The most common mt 16S rRNA gene haplotype (i.e., haplotype Is-1) comprised approximately half (49%) of the tick samples, which is consistent with that reported in earlier studies on *I. scapularis* collected from various regions in the Northeast and Southeast U.S.A. (Rich et al. 1995, Qiu et al. 2002, Van Zee et al. 2013) and southern Canada (Krakowetz et al. 2011). Two of the most common haplotypes detected in the present study (i.e., haplotypes Is-2 and Is-4) were also the second and third most commonly detected haplotypes in the study of Qiu et al. (2002). Similarly, three of the most commonly occurring haplotypes in the present study (i.e., Is-4, Is-15, and Is-13) were also commonly detected in the study of Krakowetz et al. (2011). Interestingly, six of the most commonly detected haplotypes in this study (i.e., Is-2, Is-4, Is-7, Is-13, Is-15, and Is-63) comprised 30% of the ticks collected and all of these haplotypes represented secondary or tertiary nodes in the minimum spanning network tree from which 25 (i.e., 38%) of the 66 haplotypes of the "American" clade were derived.

The prevalences of the different haplotypes differed among localities in this study as in other studies (Qiu et al. 2002, Krakowetz et al. 2011). For example, in the study by Qiu et al. (2002), the proportion of ticks representing haplotype Is-1 varied from 30% in Connecticut to 64% in Massachusetts. These results were consistent with those of the present study in that the proportion of ticks that comprised haplotype Is-1 ranged from 34% in HISK (RI) to 63% in ISP (MN). Interestingly, Qiu et al. (2002) showed that the proportion of ticks of haplotype Is-1 (i.e., 30%) was less than that for haplotype Is-4 (i.e., 48%) in Connecticut. Also, haplotype J of Qiu et al. (2002), which is the equivalent of haplotype Is-22 (Krakowetz et al. 2011), was not detected in this study and was only found in the state of New York in the study by Qiu et al. (2002).

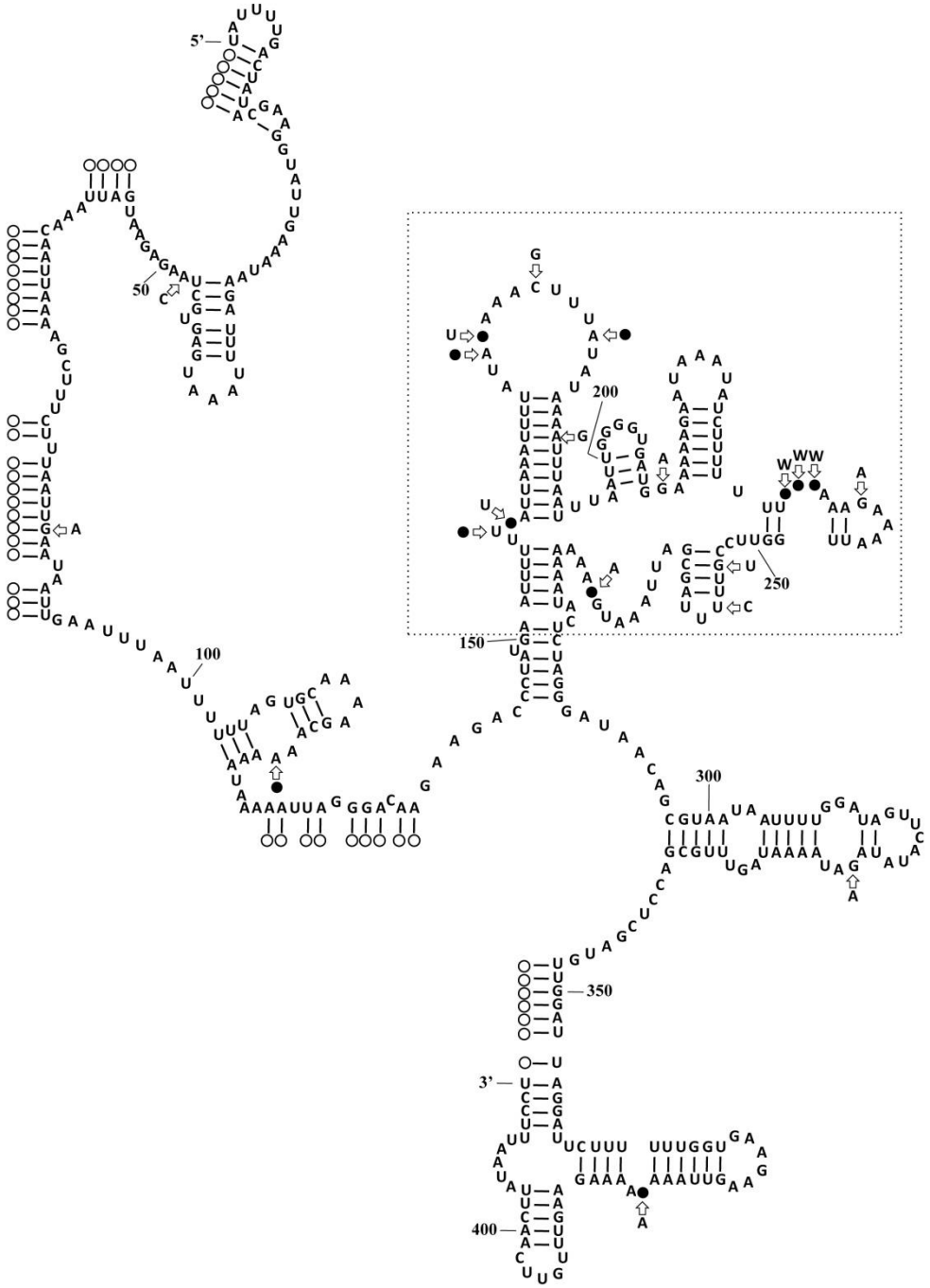


Figure 2.11. The secondary structure of the mt 16S rRNA for *I. ricinus* according to the models of Gutell and Fox (1988), Gutell et al. (1993), and Gutell (1996). Open circles indicate putative nucleotides within other regions of the mt 16S rRNA (see Gutell and Fox 1988; Gutell et al. 1993; Gutell 1996). Solid arrows indicate transition mutations, while open arrows indicate transversion, multiple, or insertion/deletion mutations relative to isolate 59 (GenBank accession no. GU074646) in the 413 bp alignment of the sequences of Noureddine et al. 2011 (GenBank accession nos. GU074588 to GU074647). The box represents the hypervariable region (e.g., Black and Piesman 1994; Misof et al. 2002, Smith and Bond 2003).

Haplotype Is-12, which comprised a large proportion of the ticks from Lunenburg, NS (i.e., 38%) (Krakowetz et al. 2011), was detected in only two tick individuals from Rhode Island in the present study. The second most common haplotype among the ticks collected from the established populations in this study differed among the populations (e.g., haplotype Is-15 in LPPP and Is-7 in HISK), and comprised between 7-17% of the ticks from each population except for LPPP and HISK where they comprised 22% and 32%, respectively. The second most common haplotype among localities in the study of Qiu et al. (2002) was generally haplotype Is-2, the proportions of which varied from 5% in Maryland to 32% in Pennsylvania. In Maryland, however, haplotype Is-6 was the second most common haplotype and comprised 32% of the 37 ticks examined (Qiu et al. 2002).

In the present study, a greater number of haplotypes were detected in the "western" as compared to the "eastern" geographical region (i.e., 33 and 26 haplotypes, respectively). Of those reported in the "western" region, 26 were not found in the "eastern" region, while 19 haplotypes were only found in the "eastern" geographical region. Furthermore, a comparison of the total number of haplotypes associated with the established populations revealed that the greatest number of haplotypes (i.e., 14 haplotypes) was detected at CR and CSP (MN), whereas the lowest number (i.e., seven haplotypes) was found at PPNP (ON) and TPSK (RI). These results suggest that there is greater genetic variability in ticks from the "western" as compared to the "eastern" geographical region. However, there was no significant difference in the total number of estimated haplotypes between the two geographical areas based on analysis of the rarefaction, extrapolation, and non-parametric estimator curves for them.

There was considerable variation in haplotype (0.60-0.79) and nucleotide (0.002-0.004) diversity among the established populations of *I. scapularis*, which was consistent with that reported previously (Krakowetz et al. 2011). There were also significant differences in the population genetic structure of *I. scapularis* based on statistical analyses of the F_{ST} data between most pairs of established populations in southern Canada, and Minnesota and Rhode Island in the U.S.A. However, there were several exceptions to this, particularly among some populations in Manitoba and Minnesota. For example, there were no significant differences in the genetic structure of tick populations in PVPP and ST in southern Manitoba, nor between CR and CSP in Minnesota. Similarly, the genetic structure of the tick population in ISP (MN) did not differ from the two populations sampled in Manitoba (i.e., ST and PVPP) nor from CSP (MN). These data

suggest that *I. scapularis* in populations in southern Manitoba are genetically more similar to those in the Upper Midwest of the U.S.A. than they are to those situated in "eastern" Canada (i.e., Ontario) or the Northeast of the U.S.A. (i.e., Rhode Island). In contrast to the absence of a significant difference in population genetic structure for the two established tick populations in southern Manitoba (i.e., PVPP and ST, which are separated by a distance of 29 km), there was a significant difference in the population genetic structure of *I. scapularis* from HISK and TPSK, two localities in Rhode Island that are separated by only 5 km, even though both populations had a similar number of haplotypes (seven and ten, respectively) and had six haplotypes in common. Chakraborty's test revealed that there was no significant difference for either population in the number of observed and expected haplotypes. Furthermore, analyses using the Chao 2 estimator suggest that the number of observed haplotypes for the TPSK population was close to the predicted number for that population, whereas the predicted number of haplotypes for the population in HISK could not be defined. Therefore, a larger sample size may need to be examined to determine if there are differences in the population genetic structures of these two populations of *I. scapularis* in Rhode Island.

The AMOVA test detected significant structuring of *I. scapularis* populations both within and among different geographical regions (i.e., provinces of Canada and states of the U.S.A.); however, the presence of shared haplotypes among populations supports the hypothesis of gene flow among them. For example, the identity and frequency of haplotypes in the *I. scapularis* populations in Manitoba and Minnesota were more similar to each other than to populations in Ontario and Rhode Island (i.e., based on the pair-wise comparisons of F_{ST} values). Furthermore, the Mantel test showed that there was a significant correlation ($r^2 = 0.30$) between the geographical (km) distances among populations and the magnitude of the genetic differences (i.e., pair-wise F_{ST} values) among them. In general, the greater the geographical distance between two populations, the greater the magnitude of the genetic difference between them. Mechai et al. (2013) found a similar correlation between the geographical distance and genetic difference based on DNA sequences of the mt cytochrome c oxidase subunit 1 (*cox1*) gene for *I. scapularis* collected from companion animals and humans, and from different locations in southern Canada. However, the significant relationship between geographical distance and genetic distance based on the mt *cox1* gene data could not be explained by linear or simple polynomial functions because the *r* value (0.026) was extremely low (Mechai et al. 2013). Factors that may contribute

to genetic differences between populations of *I. scapularis* in the absence of geographical barriers (e.g., agricultural practices) have been discussed previously (Mechai et al. 2013).

2.5.3. Phylogeography

Although the first established population of *I. scapularis* in Canada was detected in LPPP in Ontario during the early 1970's (Watson and Anderson 1976), most tick populations in southern Canada have only recently become established. For example, populations of blacklegged tick in PVPP and ST in Manitoba have only established in the past 1-2 years (L.R. Lindsay 2013, pers. comm., 3 Dec.). The recent establishment of *I. scapularis* populations in some locations in southern Canada was supported by the results of Tajima's and Fu's tests based on haplotype data of the mt LSU rRNA gene collected in the present study and in the study by Krakowetz et al. (2011). Furthermore, the results of both statistical tests on the haplotype data of ISP, CR, and CSP in Minnesota were consistent with the relatively recent establishments of *I. scapularis* populations in Minnesota during the 1980's and 1990's (e.g., Drew et al. 1988, Dennis et al. 1998, Sanders and Guilfoile 2000). It has been proposed that the westward expansion of *I. scapularis* into Minnesota is due to the migration of white-tailed deer (*Odocoileus virginianus*) carrying blacklegged ticks from established populations in Wisconsin (Riehle and Paskewitz 1996, Lee et al. 2013) and that the initial establishment of the *I. scapularis* populations in the northern Midwest of the U.S.A. (e.g., in Wisconsin) arose as the result of colonization by individuals from populations in the Northeast U.S.A. (Humphrey et al. 2010). According to Humphrey et al. (2010), colonizing individuals may have been transported to the northern Midwest from the Northeast U.S.A. as a consequence of long distance dispersal by migratory birds or, less likely, by the gradual migration of white-tailed deer through geographical regions currently supporting ticks in low densities (Diuk-Wasser et al. 2006). Migratory passerine birds have also played an important role in the dispersal of larval and nymphal *I. scapularis* into southern Canada from the U.S.A. (Klich et al. 1996, Scott et al. 2001, Morshed et al. 2005, Ogden et al. 2008b, Scott et al. 2010, Scott et al. 2012).

Differences in the population genetic structure of *I. scapularis* collected from different parts of southern Canada suggest that these ticks may have originated from tick populations in different geographical regions of the U.S.A. This hypothesis was previously proposed by Krakowetz et al. (2011) based on differences in the occurrences and frequencies of the mt 16S

rRNA gene haplotypes of *I. scapularis* among individuals within established populations in southern Manitoba, Central Canada, and the Atlantic Provinces. Support for this hypothesis has been reported by Mechai et al. (2013) whose recent study investigated the population structure of *I. scapularis* collected from within Canada using part of the mt *cox1* gene as a molecular marker. Spatial variations in the frequency of occurrence of *cox1* haplotypes that were associated with four geographical regions of Canada (i.e., Alberta to western Ontario, eastern Ontario, Quebec, and the Atlantic Provinces) were found in the study by Mechai et al. (2013), which were similar to that found in the present study and that of Krakowetz et al. (2011) for these geographical regions, except for Quebec as sample sizes from there were small (n = 7, Krakowetz et al. 2011; n = 22, this study).

In the present study, additional support for the hypothesis that there are different geographical origins for populations of *I. scapularis* in the different regions of southern Canada resulted from the pair-wise comparisons of geographical and genetic distances among the nine established populations and from Tajima's, Fu's, and Chakraborty's tests, which showed that the *I. scapularis* populations in Manitoba were most similar to those in Minnesota and not to those in Ontario or Rhode Island. Furthermore, a comparison of the mt 16S rRNA gene haplotypes found in the Prairie Provinces (i.e., Alberta, Saskatchewan, and Manitoba) to those in Minnesota revealed that most (i.e., 62%) of the 21 haplotypes detected in the Prairie Provinces were also detected in Minnesota. In contrast, only 33% and 24% of the 21 haplotypes found in the Prairie Provinces were also found in Central Canada (i.e., Ontario and Quebec) and the Atlantic provinces (i.e., Newfoundland, Nova Scotia, New Brunswick, and Prince Edward Island), respectively. Similarly, only 24% of the 25 haplotypes that were detected in Minnesota were also present in Central Canada. A comparison of the haplotypes that were detected in Minnesota to those in Rhode Island (this study) and in seven states of the Northeast U.S.A. (Qiu et al. 2002) revealed the least similarity between all pairs of regions compared; only 16% and 20% of the 25 haplotypes that were detected in Minnesota were also detected in Rhode Island and the Northeast U.S.A., respectively. Moreover, the minimum spanning network tree depicting the relationships of the mt LSU rRNA gene haplotypes belonging to the "American" clade showed a clear association between some haplotypes and the "western" geographical area only. Thus, these results suggest that *I. scapularis* in "western" Canada may be derived from colonizing individuals that originated from established populations in the northern Midwest of the U.S.A.

The Mantel and Tajima's, Fu's, and Chakraborty's tests showed that there were significant genetic differences between *I. scapularis* from established populations in Central Canada (i.e., PPNP and LPPP) as compared to Rhode Island (i.e., HISK and TPSK). Furthermore, a comparison of the mt 16S rRNA gene haplotypes detected in Central Canada to those in Rhode Island (this study) and the Northeast U.S.A. (Qiu et al. 2002) revealed that only 29% and 38% of the 21 haplotypes that were detected in Central Canada were also detected in Rhode Island and the Northeast U.S.A., respectively. Similarly, only 38% of the haplotypes found in Central Canada were also found in Atlantic Canada. In contrast, the genetic similarity of ticks collected from the Atlantic Provinces of Canada and Rhode Island in the U.S.A. was pronounced, as 78% of the 9 haplotypes detected in the Atlantic Provinces were also detected in Rhode Island. Likewise, 67% of the nine haplotypes detected in the Atlantic Provinces were previously detected in the Northeast U.S.A. by Qiu et al. (2002). Despite the dissimilarity between *I. scapularis* from Central Canada to the Atlantic Provinces of Canada and the Northeast U.S.A. (Qiu et al. 2002; this study), the minimum spanning network tree depicts a clear association between some mt 16S rRNA gene haplotypes and the "eastern" geographical area only, which includes haplotypes of ticks collected from Central Canada (Krakowetz et al. 2011; this study). Thus, these results suggest that some of the blacklegged ticks in the Atlantic Provinces and in Central Canada may have derived from colonizing individuals that originated from established populations in the Northeast of the U.S.A.

2.5.4. Conclusions

The genetic diversity in the DNA sequences of the mt 16S rRNA gene among individuals of *I. scapularis* in the northern Midwest and Northeast of the U.S.A. and in southern Canada was greater than has been documented previously (Caporale et al. 1995, Rich et al. 1995, Norris et al. 1996, Qiu et al. 2002, Trout et al. 2009, Humphrey et al. 2010, Anstead and Chilton 2011, Krakowetz et al. 2011, Van Zee et al. 2013). This may in part be a function of the larger sample sizes collected at some localities. Furthermore, the results of the statistical analyses suggest that the number of haplotypes within some populations may be underestimated, probably the result of inadequate sampling (e.g., low sample sizes) at some localities. There were also significant differences in the population genetic structure of *I. scapularis* from different localities in southern Canada, similar to that reported by Krakowetz et al. (2011) and Mechai et al. (2013) in

recent years, suggesting different U.S.A. origins for these populations. In the present study, the minimum spanning network tree (Fig. 2.9) depicted, while the diagram (Fig. 2.10) comparing the mt 16S rRNA gene haplotypes found in key geographical areas (i.e., "western" Canada, "eastern" Canada, "western" U.S.A., and "eastern" U.S.A.) provided, evidence that some of the blacklegged ticks in the Prairie Provinces may have derived from colonizing individuals that originated from established populations in the northern Midwest of the U.S.A., while some of the blacklegged ticks in the Atlantic Provinces and in Central Canada may have derived from colonizing individuals that originated from established populations in the Northeast of the U.S.A. However, approximately 60% of the blacklegged ticks collected from the established populations in southern Canada have one of four mt 16S rRNA gene haplotypes that also occur both in the northern Midwest and Northeast of the U.S.A. Thus, the geographical origins of some ticks of these haplotypes within southern Canada cannot be determined. In conclusion, the DNA sequences of Domains IV and V of the mt 16S rRNA gene provide only limited phylogeographical signal for *I. scapularis*. Additional studies are therefore needed to explore other genetic markers that may be useful for understanding the trajectories of spread of *I. scapularis* and its pathogens on a finer (e.g., regional) scale.

In the next chapter, the population genetics and phylogeographical relationships of a subset of the blacklegged ticks that were characterized in this Chapter (i.e., using Domains IV and V of the mt 16S rRNA gene) will be examined using the 3' terminal end of the mt 12S rRNA gene and 3' flanking region ($tRNA^{Val}$ gene). It will also be determined whether the concatenated sequence data provides greater resolution of the phylogeographical associations among *I. scapularis* haplotypes.

CHAPTER 3

GENETIC DIVERSITY IN THE MITOCHONDRIAL 12S RIBOSOMAL RNA GENE AND 3' FLANKING REGION (I.E., THE TRANSFER RNA^{VAL} GENE) OF *IXODES SCAPULARIS*[2]

3.1. Abstract

Genetic variation in 229 *Ixodes scapularis* was investigated using DNA sequences (~430 bp) of the 3' end of the mitochondrial (mt) 12S ribosomal (r) RNA gene and the complete transfer (t) RNA^{Val} gene. The resulting sequence data was combined with that of Domains IV and V of the mt 16S rRNA gene (Chapter 2) for each tick. Sixty-two haplotypes of the mt 12S rRNA + tRNA^{Val} genes were detected. There were 41 variable positions in the sequence alignment. Most of the mutational differences did not alter the secondary structures of the RNAs. The data analyses suggest that many more haplotypes remain to be detected. There was also strong genetic structuring based on the Analysis of Molecular Variance (AMOVA) test both within and among populations in a geographical region. Most of the genetic variation occurred within populations, a finding that was supported by the significant differences in genetic diversity between established populations of *I. scapularis* based on pair-wise F_{ST} values. Nonetheless, the presence of shared haplotypes among populations supports the hypothesis of gene flow among them. There were significant correlations between genetic (F_{ST}) and geographical (km) distances between pairs of populations based on analyses of the mt 12S rRNA + tRNA^{Val} sequence data and the concatenated sequence data. Examination of both data sets revealed an association between some tick haplotypes (based on both genetic markers) and geographical areas in Canada. However, sequences of the mt 12S rRNA + tRNA^{Val} genes lacked sufficient resolution to determine the geographical origins for 27% of the ticks from Canada, as these individuals were of a haplotype that occurred in the Upper Midwest and Northeast of the U.S.A. There was also a lack of spatial clustering of haplotypes in the minimum spanning networks, implying that these markers may not be useful for understanding the directions of

[2] Part of this chapter was reproduced with permission from Elsevier (http://www.elsevier.com/about/policies/author-agreement/lightbox_scholarly-purposes): **Krakowetz CN, Sproat A, Lindsay LR, Chilton NB (2015)** Sequence variability in the mitochondrial 12S rRNA and tRNA^{Val} genes of *Ixodes scapularis* (Acari: Ixodidae) individuals shown previously to be genetically invariant. Mol Cell Probes 29: 177-181. NBC conceived the project. LRL organized the collection of samples. CNK and AS carried out laboratory work. CNK and NBC performed the data analyses. All authors interpreted the data, wrote the manuscript, and approved the final manuscript.

spread of *I. scapularis* and its pathogens on a finer (e.g., regional) scale. Although the genetic markers examined in this chapter may provide limited phylogeographical information for *I. scapularis*, they are very useful for population genetic studies on blacklegged ticks.

3.2. Introduction

Sequences of the 3' end (i.e., Domains IV and V) of the mitochondrial (mt) 16S ribosomal (r) RNA gene have often been used to examine the population genetics and phylogeographical relationships of *Ixodes scapularis* (Norris et al. 1996, Qiu et al. 2002, Trout et al. 2009, Humphrey et al. 2010, Krakowetz et al. 2011, Van Zee et al. 2013). However, it was demonstrated in the previous chapter that this genetic marker provided only limited information regarding the potential geographical origins (i.e., in the U.S.A.) for most of the *I. scapularis* collected from different regions within Canada. This was mainly because a single haplotype (i.e., Is-1) comprised 45% of the blacklegged ticks examined from the different geographical areas in southern Canada. Therefore, a second genetic marker is needed to provide greater resolution of the phylogeographical patterns of blacklegged ticks from different regions in southern Canada.

In a study by Norris et al. (1996), parts of the mt large subunit (LSU) and small subunit (SSU) rRNA genes (i.e., the 16S and 12S rRNA genes, respectively) were used as genetic markers to compare *I. scapularis* individuals from different regions in the eastern U.S.A. Their study focused on sequence data of the mt LSU gene; however, sequences of both genes were determined for 24 ticks (Norris et al. 1996). Based on the concatenated sequence data (882 bp) of the mt 16S (462 bp) and 12S (420 bp) rRNA genes, Norris et al. (1996) revealed that there were 23 haplotypes among the 24 *I. scapularis* and that the ticks could be divided into two clades (i.e., the "American" and "southern" clades), as described in Chapter 2. Norris et al. (1996) also reported that there were nearly twice as many variable sites in the mt 12S rRNA gene of *I. scapularis* than in the mt 16S rRNA gene (i.e., 53 and 27, respectively). This variation represented 13% and 6% of the nucleotides examined for each gene, respectively. Furthermore, Norris et al. (1996) reported higher rates of base pair substitutions (i.e., transitions, transversions, and indels) among haplotypes of the mt 12S rRNA gene compared to those of the mt 16S rRNA gene. These results suggest that the 3' terminal end of the mt 12S rRNA gene may represent a more suitable genetic marker for examining the population genetics and phylogeography of *I. scapularis*.

In this chapter, the phylogeographical relationships and population genetics of *I. scapularis* from different regions of southern Canada, and the Midwest and Northeast, U.S.A. are examined using the 3' terminal end of the mt 12S rRNA gene and 3' flanking region (i.e., the transfer (t) RNA^{Val} gene). In addition, sequence data for this genetic marker are combined with the sequence data obtained for the mt 16S rRNA gene (Chapter 2) to determine if a greater resolution of the phylogeographical associations among *I. scapularis* haplotypes can be obtained.

3.3. Materials and methods

3.3.1. Samples

A total of 229 *I. scapularis* individuals were included in the present study (Table 3.1). These ticks comprised 212 individuals collected from nine established populations in Canada and the U.S.A. (Chapter 2) by drag sampling (Lindsay et al. 1999b), 15 adventitious ticks collected from cats and dogs (Chapter 2), and two individuals collected from established populations in Ontario (ON) and Nova Scotia (NS) as part of a previous study (Krakowetz et al. 2011). The DNA sequence (~400 bp) of the 3' end of the mt 16S rRNA gene (i.e., Domains IV and V) had already been determined for 228 ticks (Krakowetz et al. 2011; Chapter 2). These 228 ticks represented all of the 52 haplotypes of the mt 16S rRNA gene reported in Chapter 2 and haplotype Is-11 of Krakowetz et al. (2011).

3.3.2. Molecular analyses

Total genomic (g) DNA was extracted and purified from 227 *I. scapularis*, as described in Chapter 2, and from two ticks, as detailed in the study by Krakowetz et al. (2011). Approximately 750 bp of the mt 12S rRNA + $tRNA^{Val}$ + 16S rRNA genes was amplified from the total gDNA of 225 individual ticks by PCR using the primers Tick-12S-1 (5'-AAACTAGGA TTAGATACCC-3') (Norris et al. 1996) and Iscap-16S-New-1 (5'-ACCAGATATCATTAAAT ATG-3'), the latter of which was designed based on a comparison of DNA sequences from the mitochondrial genomes of *Ixodes ricinus* (GenBank™ accession no. JN248424) and *Ixodes persulcatus* (GenBank accession no. AB073725). These two tick species, along with *I. scapularis*, are members of the subgenus *Ixodes* (Xu et al. 2003). PCRs were conducted in

Table 3.1. Collection details of adventitious *I. scapularis* and those from the established populations.

Region	Collection year(s)	Total no. ticks	Stage† (no. ticks)	Engorgement	Host	Travel history
Adventitious						
Canada						
Prairie						
Saskatchewan (SK)	2009, 2010	3	F(3)	partial	dog	none/within province
Manitoba (MB)	2010, 2011	2	F(2)	full/partial	dog	none/local only
Central						
Ontario (ON)	2007, 2011	2	F(2)	partial	dog	local only
	2006	1[a]	adult(1)	unfed	n/a	n/a
Quebec (QC)	2005, 2010, 2011	4	F(4)	partial	cat/dog	local only/New York State*
Atlantic						
New Brunswick (NB)	2007, 2009	2	F(2)	partial	cat/dog	local only
Nova Scotia (NS)	2008, 2010	2	F(2)	partial	cat/dog	local only
	2006	1[b]	adult(1)	unfed	n/a	n/a
Total		17	F(15) adult(2)			
Established populations						
Canada						
Prairie						
Pembina Valley Provincial Park (PVPP)	2010	15	F(7) M(8)	unfed	n/a	n/a
Stanley Trail (ST)	2010	21	F(10) M(11)	unfed	n/a	n/a
Central						
Point Pelee National Park (PPNP)	2009	15	F(15)	unfed	n/a	n/a
Long Point Provincial Park (LPPP)	2000	67	F(27) M(40)	unfed	n/a	n/a

Table 3.1. Continued.

Region	Collection year(s)	Total no. ticks	Stage† (no. ticks)	Engorgement	Host	Travel history
U.S.A.						
Midwest						
Itasca State Park (ISP)	2008	13	F(8) M(5)	unfed	n/a	n/a
Camp Ripley (CR)	2008	36	F(20) M(16)	unfed	n/a	n/a
St. Croix State Park (CSP)	2008	32	F(14) M(18)	unfed	n/a	n/a
Northeast						
Hazard Island, South Kingstown (HISK)	2009, 2010	7	N(7)	unfed	n/a	n/a
Trustom Pond, South Kingstown (HISK)	2009, 2010	6	N(6)	unfed	n/a	n/a
Total		212	F(101) M(98) N(13)			

† N = nymph; adults: F = female and M = male.
* tick may have originated from an endemic area in the U.S.A.
[a] tick from the established population in PPNP, but collected in 2006 (Krakowetz et al. 2011).
[b] tick from the established population in Lunenburg (Krakowetz et al. 2011).

25 µl volumes comprised of 1X *Taq* Buffer with KCl (Fermentas, Thermo Fisher Scientific, Ottawa, ON, Canada), 200 µM of each dNTP (Fermentas), 2 mM $MgCl_2$ (Fermentas), 1 uM of each primer, 0.5 U of *Taq* DNA Polymerase (Fermentas), and 1 µl of gDNA template. A negative control consisting of UltraPure™ DNase/RNase-Free Distilled Water (Invitrogen™, Life Technologies Inc., Burlington, ON, Canada) was included in each set of PCRs. The thermocycler conditions used for the PCRs were 95ºC for 5 min, then 30 cycles of 94ºC for 1 min, 53ºC for 1 min, and 72ºC for 1 min, followed by a final 5 min extension at 72ºC. The resulting amplicons were electrophoresed at 120 V for 40 min on 1.5% (w/v) agarose-TBE gels (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3; OmniPur®, EMD Millipore Ltd., Etobicoke, ON, Canada) that were stained with SYBR® Safe DNA Gel Stain (Life Technologies Inc., Burlington, ON, Canada). Amplicons were then purified (see Chapter 2 for the methodology used) and subjected to automated DNA sequencing using the primer Tick-12S-1.

A second DNA fragment (~1800 bp) starting within the mt 12S rRNA gene, encompassing the entire $tRNA^{Val}$ and 16S rRNA genes, and ending within the NADH dehydrogenase subunit 1 gene was amplified from the total gDNA of four individual ticks by PCR using the primers Tick-12S-1 and Tick-ND1-F2 (5'-AGGAAGCTTAAATTCCT-3'). The primer Tick-ND1-F2 was designed based on comparisons of the mt DNA sequences of *I. ricinus* (GenBank accession no. JN248424) and *I. persulcatus* (GenBank accession no. AB073725). PCRs were performed in 25 µl reaction volumes consisting of 1X *Taq* Buffer with KCl (Fermentas), 2.0 mM $MgCl_2$ (Fermentas), 200 µM of each dNTP (Fermentas), 1 µM of each primer, 0.5 U of *Taq* DNA Polymerase (Fermentas), and 1 µl of gDNA template. The set of PCRs included a negative control, which consisted of UltraPure DNase/RNase-Free Distilled Water (Invitrogen). The thermocycler conditions were: 95ºC for 5 min, followed by 35 cycles at 94ºC for 1 min, 53ºC for 1 min, and 72ºC for 1 min, and finally 72ºC for 5 min. Amplicons were subjected to electrophoresis on 1.5% (w/v) agarose-TBE gels stained with SYBR® Safe DNA Gel Stain (Life Technologies) for 50 min at 140 V. Purified amplicons (see Chapter 2) were subjected to automated DNA sequencing using the primers Tick-12S-1 and Tick-ND1-F2 in separate reactions. The DNA sequences of the different 12S rRNA + $tRNA^{Val}$ gene haplotypes (~430 bp) have been deposited in GenBank under the accession numbers HG918113-HG918174.

3.3.3. Data analyses

The DNA sequences of the mt 12S rRNA + $tRNA^{Val}$ genes were aligned manually, but modified according to the predicted secondary structures of the RNAs that were constructed for *I. scapularis* based on secondary structure models for other arthropods (Gutell and Fox 1988, Gutell et al. 1993, Gutell 1996). The locations of the mutational changes in the sequence alignment were examined in relation to the secondary structures. For each tick, the sequence of the mt 12S rRNA + $tRNA^{Val}$ genes was concatenated with the sequence of Domains IV and V of the mt 16S rRNA gene. The resulting DNA sequences for 228 *I. scapularis* were aligned over 839 bp.

The computer program TCS (Clement et al. 2000) was used to generate a minimum spanning network to depict the relationships of the different haplotypes detected in this study. The computer program Arlequin (Excoffier and Lischer 2010) was used to estimate haplotype and nucleotide diversities among ticks from nine established populations and to conduct the following tests of neutrality: Tajima's *D* (Tajima 1989), Fu's F_S (Fu 1997), and Chakraborty's test (Chakraborty 1990). Arlequin was also used to calculate measures of genetic differentiation between pairs of populations (i.e., F_{ST} values) and to conduct a Mantel test using 1000 permutations to determine if there was a correlation between genetic (F_{ST}) and geographical (km) distances. A hierarchical Analysis of Molecular Variance (AMOVA) test was conducted using Arlequin to estimate the genetic diversity within and among populations in different geographical regions. For this analysis, the nine established populations of *I. scapularis* were divided into four groups based on the provinces (Canada) or states (U.S.A.) in which they were located. All of these analyses were also performed on a concatenated data set; that is, the DNA sequences of the mt 12S rRNA + $tRNA^{Val}$ genes combined with the sequences of Domains IV and V of the mt 16S rRNA gene for the 229 ticks (Chapter 2).

A phylogenetic analysis using the neighbour-joining (NJ) method was carried out on the sequence data using the computer program PAUP (Swofford 2002). The DNA sequence of the mt 12S rRNA + $tRNA^{Val}$ genes of *I. ricinus* (GenBank accession no. JN248424) was used as the outgroup. A bootstrap analysis (1000 replicates) was conducted to determine the relative support for branches in the phylogenetic tree. These analyses were also performed on the concatenated data set.

Rarefaction (i.e., interpolation) curves and their 95% confidence intervals based on sample-based abundance data (i.e., haplotype frequency data) for the "eastern" (i.e., Point Pelee National Park (PPNP) and Long Point Provincial Park (LPPP) in ON; and Trustom Pond, South Kingstown (TPSK) and Hazard Island, South Kingstown (HISK) in Rhode Island (RI)) and "western" (i.e., Pembina Valley Provincial Park (PVPP) and Stanley Trail (ST) in Manitoba (MB), and Itasca State Park (ISP), Camp Ripley (CR), and St. Croix State Park (CSP) in Minnesota (MN)) geographical regions were generated using the computer program EstimateS (Colwell 2013). These curves were made to estimate the number of expected haplotypes when between one and *n* ticks are "collected" (i.e., characterized based on their sequences of the mt 12S rRNA + $tRNA^{Val}$ genes), where *n* is the total number of ticks from each geographical area (e.g., 117 for "western" and 96 for "eastern"). Extrapolation curves and their 95% confidence intervals were generated using the same program and data in order to estimate the number of expected haplotypes when between *n* and 2*n* ticks are "collected" (or characterized). The total haplotype richness for the "western" region between one and *n* was also estimated using EstimateS and the bias-corrected version of Chao 2 with 1000 randomizations, whereas the total haplotype richness for the "eastern" region between one and *n* was estimated using the classic version of Chao 2 with 1000 randomizations, as was recommended for these datasets by the program. An additional set of rarefaction and extrapolation curves and their respective 95% confidence intervals were constructed using EstimateS and the sample-based abundance data for the nine established populations. The total haplotype richness between one and *n*, where *n* is the total number of ticks from the established populations, was estimated using EstimateS and the classic version of Chao 2 with 1000 randomizations. A scatter plot of the number of haplotypes observed at each established population as a function of the total number of ticks collected from that population was also created and overlaid upon the rarefaction and extrapolation curves for the nine established populations. These analyses were also performed on the concatenated data set.

3.4. Results

3.4.1. Mt 12S rRNA + tRNA^{Val} genes

Amplicons of the mt 12S rRNA gene, the entire mt tRNA^{Val} gene, and the 5' end (i.e., Domains I and II) of the mt 16S rRNA gene produced a single band of the expected size (i.e., ~700 bp) on agarose gels upon electrophoresis (not shown). Bands were not detected for the negative control samples. Based on sequences of the mt 12S rRNA + tRNA^{Val} genes, 62 haplotypes were detected among the 229 ticks (Table 3.2). The DNA sequences of the haplotypes varied in length from 424-428 bp, while the haplotypes differed from one another by 1-13 bp when aligned over 430 bp. There were 41 variable positions in the sequence alignment of the mt 12S rRNA + tRNA^{Val} genes, four of which represented purine transitions and nine of which represented pyrimidine transitions. There were also 15 transversions, three insertions/deletions (i.e., indels), and 10 multiple mutational changes in the alignment. The ratio of transitions to transversions was approximately 1:1.

The locations of these mutational changes in relation to the predicted secondary structures of the RNAs are shown in Fig. 3.1. Thirty-seven (i.e., 90%) of the variable positions in the sequence alignment occurred in unpaired regions (i.e., end loops or bulges of helices), and, thus, did not alter the secondary structures of either the rRNA or the tRNA. However, mutational changes at each of the remaining variable positions in the alignment altered the pairing arrangements of helices in the secondary structure of the rRNA. These mutational changes were comprised of two transversions at positions 43 and 120, one pyrimidine transition at position 206, and one multiple mutational change at position 133. The multiple mutational change resulted from a purine transition in the sequence of haplotype CT22, which represented a partial-compensatory mutational change (i.e., a mutation on one side of a helix that is associated with the maintenance of base pairing), and a transversional change in the sequence of haplotype CT02, which represented a non-compensatory mutational change resulting in the formation of an inner bulge on a helical structure. The other mutational changes affecting the pairing arrangements of helices in the secondary structure of the rRNA represented non-compensatory mutational changes, which resulted in the formation of an inner bulge on a helical structure (see positions 43, 120, and 206).

Table 3.2. The number of *I. scapularis* individuals of each haplotype (HT) of the mt 12S rRNA + $tRNA^{Val}$ genes, and the variable positions in the 430 bp alignment of the DNA sequences for this region of the mitochondrial genome. A dot (.) at an alignment position indicates the same nucleotide as in the sequence of haplotype CT08, while a dash (-) represents a deletion.

| HT | *n* | Alignment position[a]: | | |

Table 3.2. Continued.

| HT | *n* | Alignment position[a]: | | |

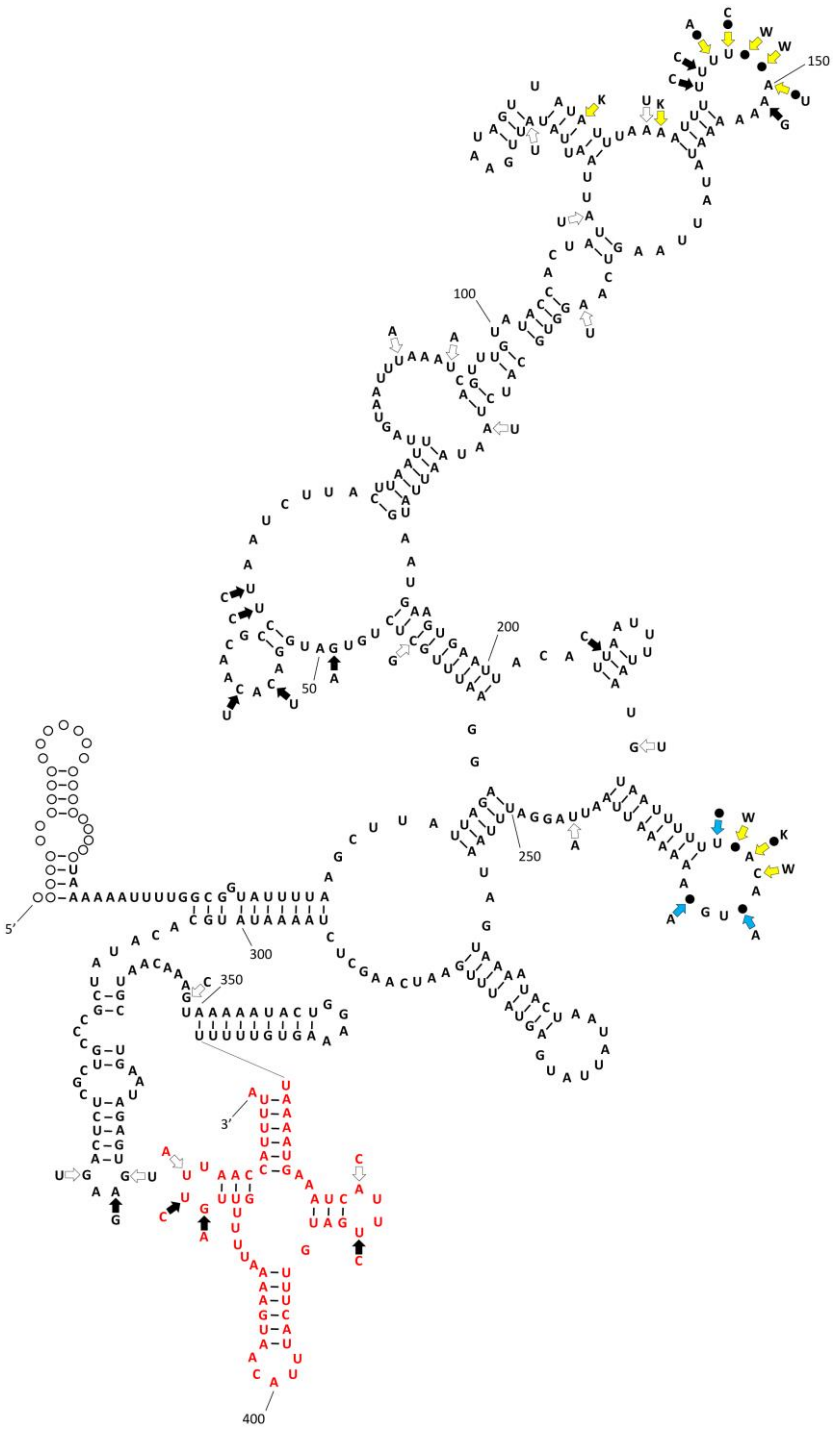


Figure 3.1. The secondary structures of the mt 12S rRNA (black characters) and tRNA^{Val} (red characters) for *I. scapularis* according to the models of Gutell and Fox (1988), Gutell et al. (1993), and Gutell (1996). Open circles indicate putative nucleotides within other regions of the mt 12S rRNA (see Gutell and Fox 1988; Gutell et al. 1993; Gutell 1996). Mutations relative to haplotype CT08 in the 430 bp alignment are shown. Black arrows indicate transitions, while white arrows indicate transversions. Multiple mutational changes are indicated by blue arrows, whereas yellow arrows indicate insertion/deletion mutations.

The most commonly detected haplotype of the mt 12S rRNA + $tRNA^{Val}$ genes in this study was CT08, which comprised 24.9% of the 229 ticks and was found in four of the eight geographical regions sampled from (i.e., MB, MN, ON, and RI) (Table 3.3). The second most common haplotype (i.e., CT07) comprised 14.8% of the ticks and was detected in only three geographical regions (i.e., MB, MN, and ON). In contrast, haplotype CT06, which represented only 5.2% of the ticks, was found in five geographical regions. Approximately half (i.e., 30) of the haplotypes were each represented by a single tick (i.e., singletons). Furthermore, 46 haplotypes were only found in a single geographical area.

The number of haplotypes detected in each of the nine established populations of *I. scapularis* in southern Canada and the northern U.S.A. varied from 4-17 (Fig. 3.2). Haplotype CT08 was found in every population, except for HISK. The most frequently detected haplotypes in the established populations with samples sizes of greater than 30 were haplotype CT07 in LPPP (ON), which comprised 40.3% of the ticks from there, and haplotype CT08 in CR and CSP (MN), which represented 41.7% and 25.0% of the ticks from each population, respectively.

A comparison of the mt 12S rRNA + $tRNA^{Val}$ gene haplotypes of 82 ticks that were previously characterized as haplotype Is-1, based on sequence comparisons of ~400 bp (i.e., Domains IV and V) of the mt 16S rRNA gene (Krakowetz et al. 2011; Chapter 2), is shown in Fig. 3.3. The ticks represented a total of 31 haplotypes, the most common of which was CT08. Haplotypes CT05, CT30, CT06, and CT25 were each represented by 3-7 ticks, while the remaining 26 haplotypes were each represented by one or two tick individuals. A total of three haplotypes were detected in both the "western" and "eastern" regions (as defined in Chapter 2), whereas 21 and seven haplotypes were found only in the "western" and "eastern" regions, respectively. The most common haplotype in LPPP (ON) was CT25, which comprised 37.5% of the ticks from there, while the most common haplotype in CSP and CR (MN) was CT08, which comprised 22.2% and 36.8% of the ticks from these localities, respectively. Remarkably, all seven ticks of haplotype Is-1 based on their mt 16S rRNA gene sequences that were collected from PPNP (ON) represented haplotype CT08 based on their sequences of the mt 12S rRNA + $tRNA^{Val}$ genes.

The haplotype and nucleotide diversities of *I. scapularis* collected from the nine established populations varied from 0.6917 to 0.9333 and 0.004965 to 0.015055, respectively (Table 3.4). Chakraborty's test showed that significantly more haplotypes were detected than

Table 3.3. The number of *I. scapularis* from eight geographical localities in North America of each haplotype of the mt 12S rRNA + $tRNA^{Val}$ genes.

Haplotype	*n*	No. individuals from:							
		SK	MB	MN	ON	QC	RI	NB	NS
CT01	2	0	0	0	2	0	0	0	0
CT02	6	0	0	0	6	0	0	0	0
CT03	2	0	0	2	0	0	0	0	0
CT04	1	0	0	0	1	0	0	0	0
CT05	4	0	0	3	1	0	0	0	0
CT06	12	0	1	2	6	0	2	0	1
CT07	34	0	3	3	28	0	0	0	0
CT08	57	0	8	28	20	0	1	0	0
CT09	1	0	0	1	0	0	0	0	0
CT10	2	0	0	2	0	0	0	0	0
CT11	4	0	2	2	0	0	0	0	0
CT12	1	0	1	0	0	0	0	0	0
CT13	2	0	0	2	0	0	0	0	0
CT14	2	0	0	0	2	0	0	0	0
CT15	1	0	1	0	0	0	0	0	0
CT16	2	0	0	0	2	0	0	0	0
CT17	1	0	1	0	0	0	0	0	0
CT18	1	0	1	0	0	0	0	0	0
CT19	1	0	1	0	0	0	0	0	0
CT20	2	0	0	2	0	0	0	0	0
CT21	3	0	1	0	2	0	0	0	0
CT22	1	0	0	0	1	0	0	0	0
CT23	5	0	3	1	1	0	0	0	0
CT24	2	0	1	1	0	0	0	0	0
CT25	8	0	0	2	6	0	0	0	0
CT26	1	1	0	0	0	0	0	0	0
CT27	6	0	0	6	0	0	0	0	0
CT28	6	0	5	1	0	0	0	0	0
CT29	6	1	5	0	0	0	0	0	0
CT30	3	0	0	3	0	0	0	0	0
CT31	3	0	0	3	0	0	0	0	0
CT32	2	0	1	1	0	0	0	0	0
CT33	1	0	1	0	0	0	0	0	0
CT34	3	0	0	0	0	0	3	0	0
CT35	1	0	0	0	0	0	0	1	0
CT36	1	0	0	0	0	1	0	0	0
CT37	2	0	0	0	0	0	0	1	1
CT38	3	0	0	0	3	0	0	0	0
CT39	1	0	0	0	0	0	1	0	0
CT40	3	0	0	0	1	0	2	0	0
CT41	1	0	1	0	0	0	0	0	0
CT42	1	0	0	0	0	0	0	0	1
CT43	3	0	0	3	0	0	0	0	0
CT44	1	0	0	1	0	0	0	0	0
CT45	3	0	0	1	0	1	1	0	0
CT46	1	0	0	0	0	1	0	0	0
CT47	2	1	0	1	0	0	0	0	0
CT48	1	0	0	1	0	0	0	0	0
CT49	1	0	0	1	0	0	0	0	0
CT50	1	0	0	1	0	0	0	0	0
CT51	1	0	0	0	0	1	0	0	0
CT52	1	0	0	1	0	0	0	0	0
CT53	1	0	0	1	0	0	0	0	0
CT54	1	0	0	0	0	0	1	0	0
CT55	1	0	0	0	1	0	0	0	0
CT56	3	0	0	3	0	0	0	0	0
CT57	1	0	0	1	0	0	0	0	0
CT58	1	0	1	0	0	0	0	0	0
CT59	1	0	0	0	1	0	0	0	0
CT60	1	0	0	1	0	0	0	0	0
CT61	2	0	0	0	0	0	2	0	0
CT62	1	0	0	0	1	0	0	0	0
Total	229	3	38	81	85	4	13	2	3

A. Manitoba

Haplotype	*n*	No. individuals from:	
		PVPP	ST
CT06	1	1	0
CT07	3	3	0
CT08	8	2	6
CT11	2	0	2
CT15	1	0	1
CT17	1	1	0
CT18	1	1	0
CT19	1	0	1
CT21	1	1	0
CT23	3	0	3
CT24	1	0	1
CT28	5	0	5
CT29	4	3	1
CT32	1	1	0
CT33	1	1	0
CT41	1	0	1
CT58	1	1	0
Total	36	15	21

B. Ontario

Haplotype	*n*	No. individuals from:	
		PPNP*	LPPP
CT01	2	0	2
CT02	6	0	6
CT04	1	1	0
CT05	1	0	1
CT06	6	1	5
CT07	28	1	27
CT08	18	9	9
CT14	2	0	2
CT16	2	0	2
CT21	2	2	0
CT22	1	1	0
CT23	1	1	0
CT25	6	0	6
CT38	3	0	3
CT40	1	0	1
CT55	1	0	1
CT59	1	0	1
CT62	1	0	1
Total	83	16	67

* Includes the tick collected in 2006 from PPNP (Krakowetz et al. 2011).

C. Minnesota

Haplotype	*n*	No. individuals from:		
		ISP	CR	CSP
CT03	2	1	1	0
CT05	3	0	3	0
CT06	2	0	0	2
CT07	3	1	1	1
CT08	28	5	15	8
CT09	1	0	0	1
CT10	2	0	0	2
CT11	2	1	0	1
CT13	2	2	0	0
CT20	2	0	0	2
CT23	1	1	0	0
CT24	1	0	1	0
CT25	2	0	1	1
CT27	6	0	1	5
CT28	1	0	1	0
CT30	3	0	1	2
CT31	3	0	3	0
CT32	1	0	1	0
CT43	3	2	0	1
CT44	1	0	1	0
CT45	1	0	0	1
CT47	1	0	0	1
CT48	1	0	0	1
CT49	1	0	0	1
CT50	1	0	1	0
CT52	1	0	0	1
CT53	1	0	1	0
CT56	3	0	3	0
CT57	1	0	1	0
CT60	1	0	0	1
Total	81	13	36	32

D. Rhode Island

Haplotype	*n*	No. individuals from:	
		TPSK	HISK
CT06	2	2	0
CT08	1	1	0
CT34	3	2	1
CT39	1	0	1
CT40	2	0	2
CT45	1	1	0
CT54	1	0	1
CT61	2	0	2
Total	13	6	7

Figure 3.2. The number of *I. scapularis* of the different haplotypes of the mt 12S rRNA + $tRNA^{Val}$ genes collected from: A) PVPP = Pembina Valley Provincial Park and ST = Stanley Trail; B) PPNP = Point Pelee National Park and LPPP = Long Point Provincial Park; C) ISP = Itasca State Park, CR = Camp Ripley, and CSP = St. Croix State Park; and, D) TPSK = Trustom Pond and HISK = Hazard Island.

A. Manitoba

Haplotype	*n*	No. individuals from:	
		PVPP	ST
CT06	1	1	0
CT08	7	1	6
CT15	1	0	1
CT19	1	0	1
CT21	1	1	0
CT33	1	1	0
CT58	1	1	0
Total	13	5	8

B. Ontario

Haplotype	*n*	No. individuals from:	
		PPNP	LPPP
CT06	2	0	2
CT07	1	0	1
CT08	11	7	4
CT25	6	0	6
CT55	1	0	1
CT59	1	0	1
CT62	1	0	1
Total	23	7	16

C. Minnesota

Haplotype	*n*	No. individuals from:		
		ISP	CR	CSP
CT03	2	1	1	0
CT05	3	0	3	0
CT06	2	0	0	2
CT08	14	3	7	4
CT09	1	0	0	1
CT10	2	0	0	2
CT20	2	0	0	2
CT25	1	0	0	1
CT27	1	0	0	1
CT30	3	0	1	2
CT32	1	0	1	0
CT44	1	0	1	0
CT47	1	0	0	1
CT49	1	0	0	1
CT50	1	0	1	0
CT52	1	0	0	1
CT53	1	0	1	0
CT56	2	0	2	0
CT57	1	0	1	0
Total	41	4	19	18

D. Rhode Island

Haplotype	*n*	No. individuals from:	
		TPSK	HISK
CT08	1	1	0
CT45	1	1	0
CT54	1	0	1
CT61	2	0	2
Total	5	2	3

Figure 3.3. The number of *I. scapularis* of the different haplotypes of the mt 12S rRNA + $tRNA^{Val}$ genes that correspond to the mt 16S rRNA gene haplotype of Is-1 (GenBank accession no. FR799011) collected from: A) PVPP = Pembina Valley Provincial Park and ST = Stanley Trail; B) PPNP = Point Pelee National Park and LPPP = Long Point Provincial Park; C) ISP = Itasca State Park, CR = Camp Ripley, and CSP = St. Croix State Park; and, D) TPSK = Trustom Pond and HISK = Hazard Island.

Table 3.4. Haplotype diversity (h) and nucleotide diversity (π) estimates, and tests of neutrality within the mt 12S rRNA + $tRNA^{Val}$ genes of *I. scapularis* from nine established tick populations in North America.

Site[a]	n	No. polymorphic sites	h	π	Tajima's test		Fu's test		Chakraborty's test		
					Tajima's D	p^b	*Fs*	p^b	No. haplotypes: Exp.	Obs.	p^b
PVPP	15	8	0.9333	0.007588	-0.59983	0.28600	-3.79535	0.01200	10.13335	10	0.22798
ST	21	11	0.8619	0.005197	-0.94100	0.20500	-2.73029	0.05100	8.78817	9	0.19207
ISP	13	11	0.8462	0.008159	-1.22752	0.13200	-0.68671	0.31400	6.49207	7	0.22800
CR	36	21	0.8190	0.005810	-2.01361	0.00500	-8.41744	0.00000	9.14841	16	0.00316
CSP	32	19	0.9153	0.007938	-1.17819	0.13100	-7.99866	0.00000	14.34864	17	0.08978
PPNP[c]	16	11	0.6917	0.004965	-1.37217	0.08200	-1.53077	0.13800	4.49905	7	0.06854
LPPP	67	21	0.8042	0.008770	-1.57898	0.04300	-1.21940	0.35400	10.51674	14	0.05969
TPSK	6	8	0.8667	0.008081	-0.05002	0.43600	0.49779	0.56100	4.30791	4	0.34437
HISK	7	13	0.9048	0.015055	0.27043	0.62500	0.78223	0.62100	5.29542	5	0.32944

[a] Ticks from MB: PVPP = Pembina Valley Provincial Park and ST = Stanley Trail; MN: ISP = Itasca State Park, CR = Camp Ripley, and CSP = St. Croix State Park; ON: PPNP = Point Pelee National Park and LPPP = Long Point Provincial Park; and South Kingstown, RI: TPSK = Trustom Pond and HISK = Hazard Island.

[b] Significance levels are $p < 0.02$ for Fu's test and $p < 0.05$ for Tajima's and Chakraborty's tests.

[c] Includes the tick collected in 2006 from PPNP (Krakowetz et al. 2011).

would be expected under neutrality for one population in Manitoba (i.e., CR). There were no significant departures from the expected number of haplotypes in all other populations sampled. Tajima's and/or Fu's tests indicated that there were significant negative departures from zero for four populations (i.e., PVPP, CR, CSP, and LPPP). Genetic distances (i.e., F_{ST} values) between pairs of *I. scapularis* populations were statistically significant for most pairs (Table 3.5). The Mantel test revealed a statistically significant correlation ($b = 0.000042$, $r^2 = 0.108$, $p = 0.042$) between geographical (km) and genetic (pair-wise F_{ST}) distances (Fig. 3.4).

The curves generated using the non-parametric estimator Chao 2 indicated that the total numbers of haplotypes in the "western" and "eastern" established populations was 59 and 64, respectively (not shown). In contrast, the interpolation (i.e., rarefaction) and extrapolation curves for the "western" and "eastern" populations showed that the estimated number of haplotypes in the "western" populations was greater than that in the "eastern" populations; however, this difference was not statistically significant, because the 95% confidence intervals of the curves overlapped (Fig. 3.5). The rarefaction and extrapolation curves for the "western" and "eastern" populations did not reach an asymptote (Fig. 3.5). The interpolation and extrapolation curves for the combined population data also failed to converge on an asymptote (Fig. 3.6). The Chao 2 estimate for the true number of haplotypes in the nine established populations was 96. The scatter plot of the total number of haplotypes detected in each population against the total number of ticks collected from each population showed that more haplotypes were detected in each of the populations in the "western" and "eastern" geographical areas than would be expected for a given sample size (i.e., each point laid on or above the curve), except for in the population in LPPP (ON) (Fig. 3.6). In LPPP, fewer haplotypes were detected than would be expected for the sample size of 67 ticks (i.e., the point corresponding to LPPP laid beyond the 95% confidence interval).

The AMOVA test indicated strong genetic structuring within and among populations in a geographical region (Table 3.6). There was no evidence of genetic structuring among populations of different geographical regions (i.e., provinces of Canada or states of the U.S.A.); however, most of the genetic variation (i.e., 91%) occurred within populations. The tree produced from the phylogenetic (NJ) analyses had limited resolution and little or no statistical support (i.e., bootstrap values <50%) for different branches in the tree (not shown).

Table 3.5. Pair-wise comparisons of geographical (km; upper diagonal) and genetic (F_{ST} values; lower diagonal) distances based on sequences of the mt 12S rRNA + $tRNA^{Val}$ genes among nine established populations of *I. scapularis* in North America.

	PVPP[a]	ST	ISP	CR	CSP	PPNP[b]	LPPP	TPSK	HISK
PVPP	--	29	307	426	543	1452	1554	2241	2246
ST	0.08985***	--	326	447	560	1465	1565	2247	2252
ISP	-0.00096	0.06310*	--	123	238	1157	1272	1978	1983
CR	0.08644**	0.00803	0.06059*	--	145	1061	1186	1904	1909
CSP	0.06757***	0.03455***	0.05603*	0.00610	--	921	1042	1758	1763
PPNP	0.02863	0.01475	0.03254	-0.00318	0.01330	--	188	909	914
LPPP	0.06012	0.23738***	0.14125***	0.24279***	0.20871***	0.19107***	--	738	743
TPSK	0.00964	0.17838**	-0.01425	0.17697***	0.09536*	0.10815	0.12778*	--	5
HISK	0.17465*	0.27512***	0.14220**	0.29338***	0.22544***	0.24404**	0.23587***	0.09568	--

*** Significance level $P < 0.001$.
** Significance level $P < 0.01$.
* Significance level $P < 0.05$.
[a] MB: PVPP = Pembina Valley Provincial Park and ST = Stanley Trail; MN: ISP = Itasca State Park, CR = Camp Ripley, and CSP = St. Croix State Park; ON: PPNP = Point Pelee National Park and LPPP = Long Point Provincial Park; and South Kingstown, RI: TPSK = Trustom Pond and HISK = Hazard Island.
[b] Includes the tick collected in 2006 from PPNP (Krakowetz et al. 2011).

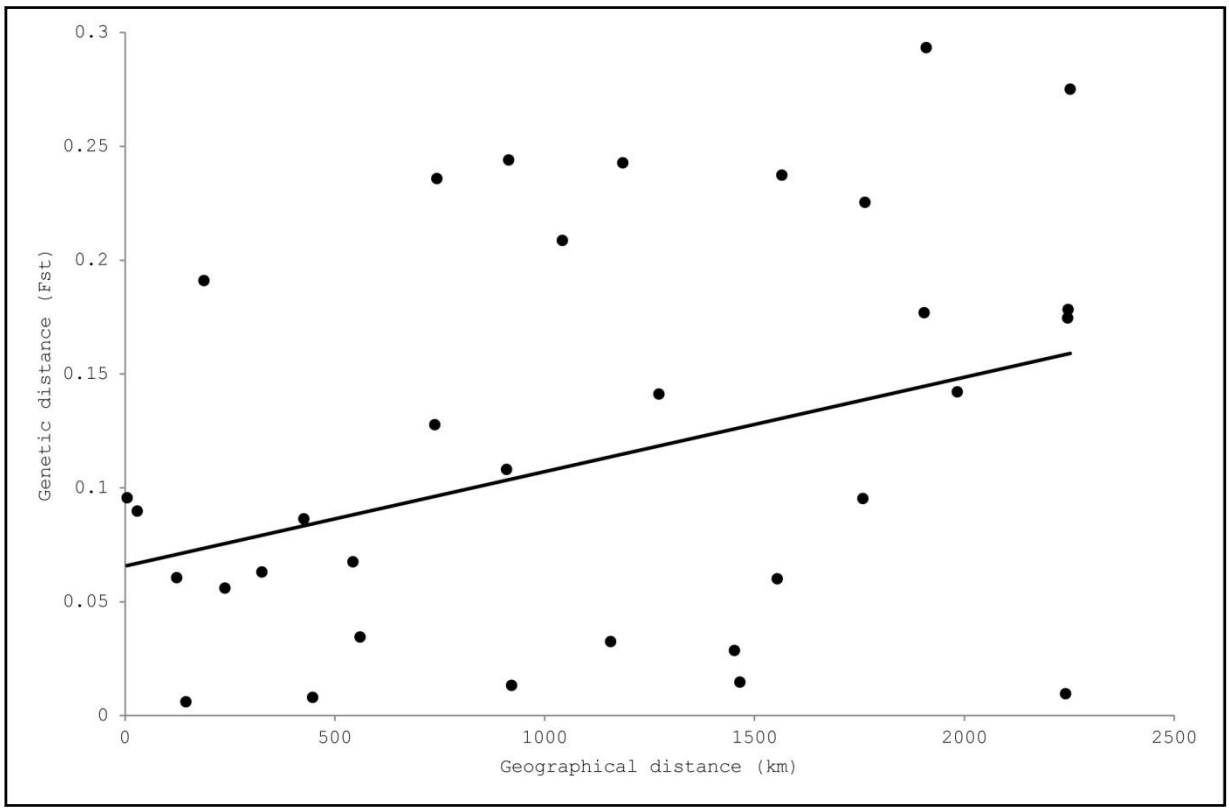


Figure 3.4. Scatter plot depicting the pair-wise comparisons of genetic (F_{ST}) and geographical (km) distances among the nine established populations of *I. scapularis* in Canada and the U.S.A. Genetic distances were determined using the data of the mt 12S rRNA + $tRNA^{Val}$ genes.

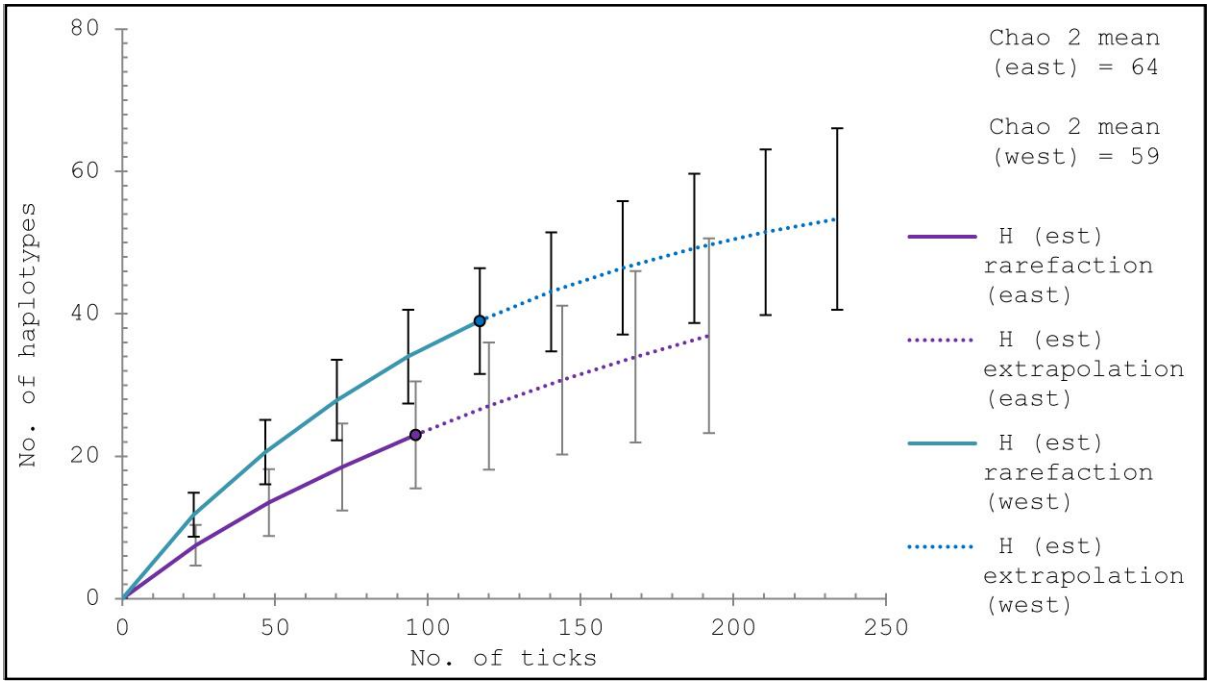


Figure 3.5. Rarefaction and extrapolation curves with 95% confidence intervals based on the data of the mt 12S rRNA + $tRNA^{Val}$ genes for the five established populations of *I. scapularis* in the "western" (Pembina Valley Provincial Park, Stanley Trail, Itasca State Park, Camp Ripley, and St. Croix State Park) and the four in the "eastern" (Point Pelee National Park, Long Point Provincial Park, Trustom Pond, South Kingstown, and Hazard Island, South Kingstown) areas of the U.S.A. and Canada. The total haplotype richness for each of the "western" and "eastern" regions (i.e., 59 and 64 haplotypes, respectively) was estimated using Chao 2 (resulting curves not shown).

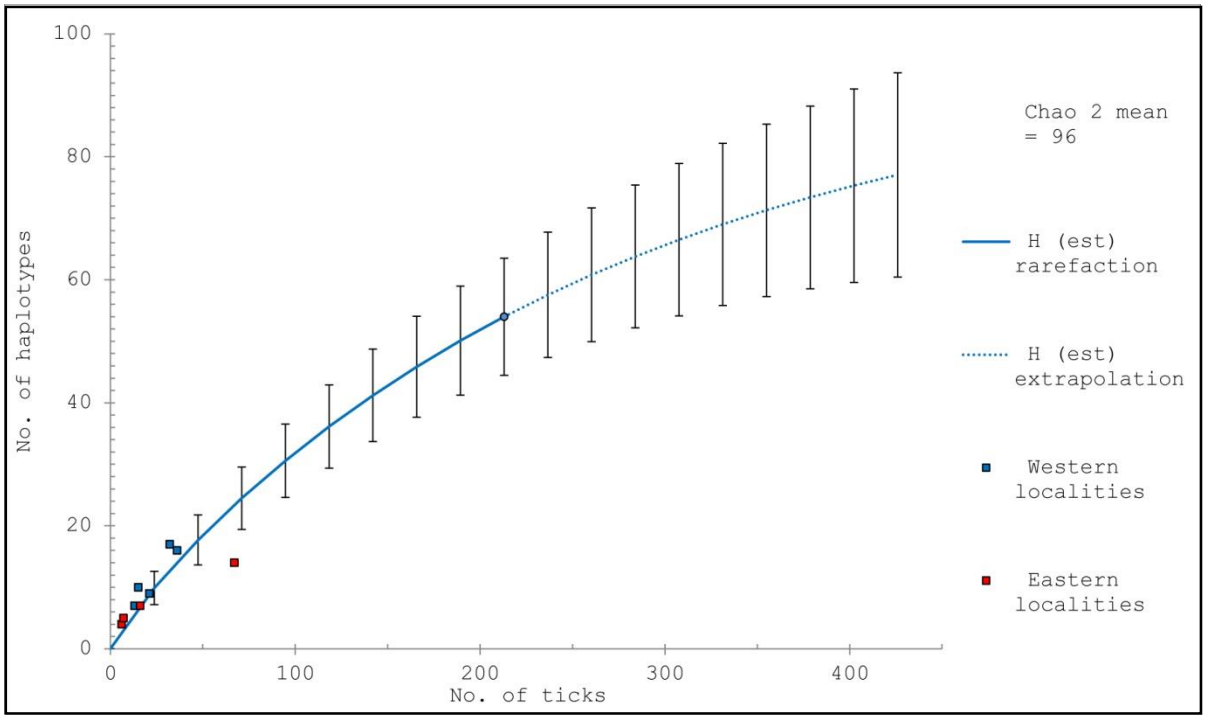


Figure 3.6. Rarefaction and extrapolation curves with 95% confidence intervals based on the data of the mt 12S rRNA + $tRNA^{Val}$ genes for the nine established populations of *I. scapularis* in the U.S.A. and Canada, and scatter plot of the number of haplotypes found at each population against the total number of ticks collected from each (see Fig. 3.2 for data). The total haplotype richness for all nine populations (i.e., 96 haplotypes) was estimated using Chao 2 (resulting curve not shown).

Table 3.6. Analysis of Molecular Variance (AMOVA) for nine established populations of *I. scapularis* from Canada and the U.S.A. based on the data of the mt 12S rRNA + $tRNA^{Val}$ genes.

Variance component	*df*	% variance	Fixation index	*p*
Among regions[a]	3	2.4	Φ_{CT} = 0.02363	> 0.05
Among populations within regions	5	6.5	Φ_{SC} = 0.06664	< 0.0001
Within populations	204	91.1	Φ_{ST} = 0.08870	< 0.0001

[a] Regions = Manitoba (PVPP, ST), Ontario (PPNP, LPPP), Minnesota (ISP, CR, CSP) and Rhode Island (TPSK, HISK).

Fig. 3.7 depicts the relationships among the 62 haplotypes of the mt 12S rRNA + $tRNA^{Val}$ genes. The haplotypes of the network differed from one another by 1-16 bp. The pattern of the minimum spanning network was complex, consisting of a "star" pattern where haplotype CT08 represented a central haplotype from which 13 haplotypes directly differed by 1-2 bp. Two other patterns were evident. The first consisted of chains of known or putative haplotypes (i.e., ≥ three haplotypes in length and no branching), and the second consisted of central haplotypes for neighbouring haplotypes (i.e., "starburst" patterns). For example, haplotype CT33 (which directly differed from haplotype CT08 by 1 bp), as well as haplotypes CT46 and CT22, were representative of the "chain" pattern, whereas haplotype CT23 (which also directly differed from haplotype CT08 by 1 bp), in addition to haplotypes CT10, CT29, CT21, CT47, CT45, and CT25 (which differed from haplotype CT23 by 1 or 3 bp), were representative of the "starburst" pattern. The second most commonly detected haplotype of these genes (i.e., CT07) represented a terminal node in the network, despite differing from haplotype CT08 by only 4 bp (through haplotypes CT18 and CT56). All haplotypes that differed from haplotype CT08 by 1-2 bp, except for haplotype CT46, were represented by ticks that were collected from localities in the "western" geographical region, or from localities in both of the "western" and "eastern" geographical regions. In contrast, haplotypes detected in only the "eastern" geographical region, and some of those from both the "western" and "eastern" regions, were linked to haplotype CT08 through haplotypes represented by ticks from the "western" or both the "western" and "eastern" geographical regions. Haplotypes detected only in the "eastern" region differed from haplotype CT08 by at least 2 bp.

Fig. 3.8 shows the number of haplotypes of the mt 12S rRNA + $tRNA^{Val}$ genes that were detected in each of four geographical areas: "western" Canada, "eastern" Canada, "western" U.S.A., and "eastern" U.S.A. The number of haplotypes detected in the "western" geographical areas of Canada and the U.S.A. (i.e., 41 haplotypes; $n = 122$) was greater than the number detected in the "eastern" geographical areas (i.e., 29 haplotypes; $n = 107$). The number of haplotypes in common between "western" Canada and "western" U.S.A. (i.e., nine haplotypes) was approximately twice as great as the number in common between "eastern" Canada and "eastern" U.S.A. (i.e., four haplotypes). Blacklegged ticks collected from localities in southern Canada represented 40 haplotypes ($n = 135$), while those collected from localities in northern U.S.A. represented 35 haplotypes ($n = 94$). The number of haplotypes in common between

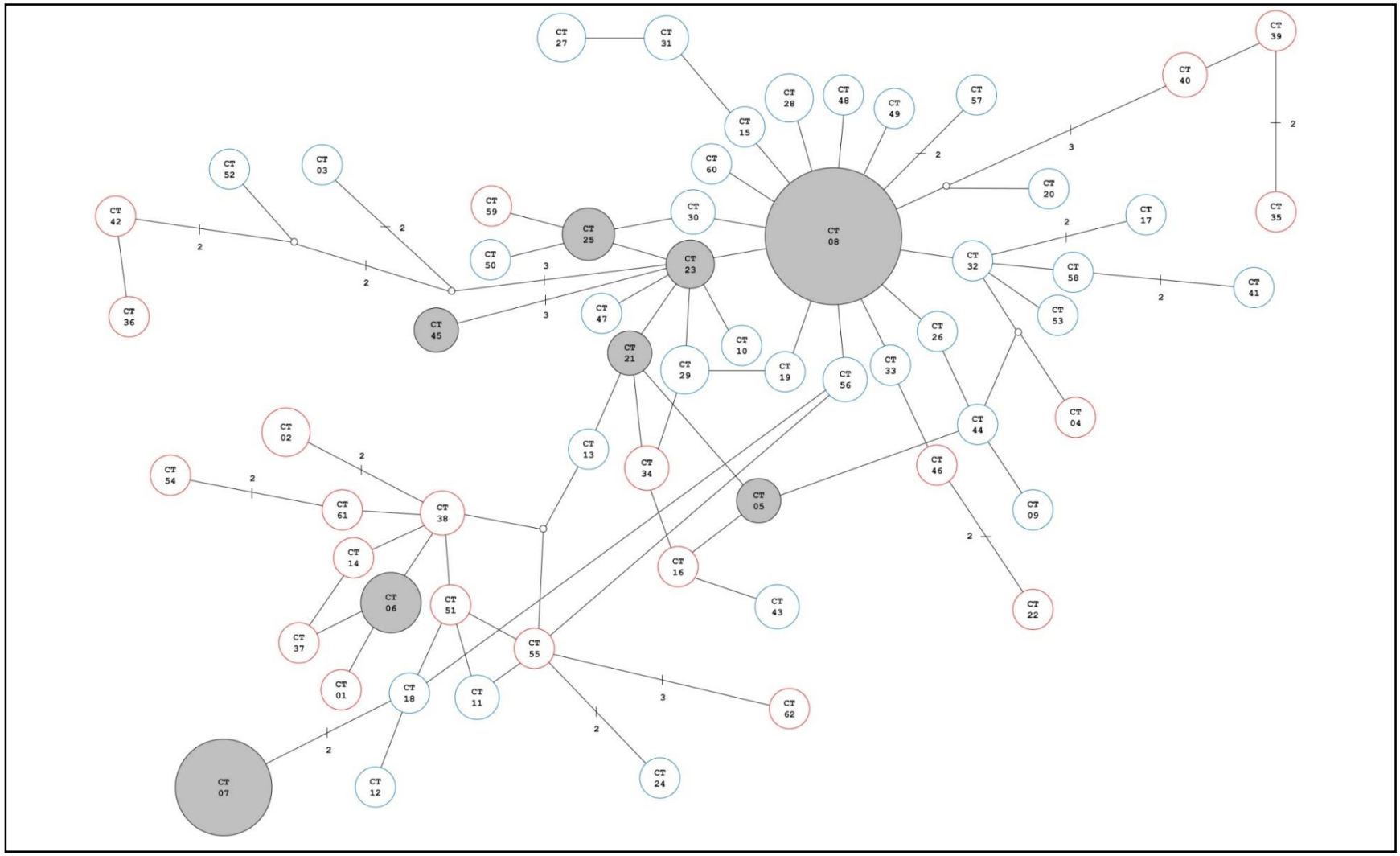


Figure 3.7. Minimum spanning network depicting the relationships among the haplotypes of the mt 12S rRNA + tNRA^{Val} genes of *I. scapularis*. The number of nucleotide differences between pairs of DNA sequences (circles) is one unless otherwise indicated. The size of each circle is proportional to the number of ticks of that haplotype. Putative haplotypes are represented by small open circles. Blue, grey, or red circles denote the different haplotypes (CT01-CT62) depending on whether each was detected in the "western," "western" and "eastern," or "eastern" geographical areas, respectively.

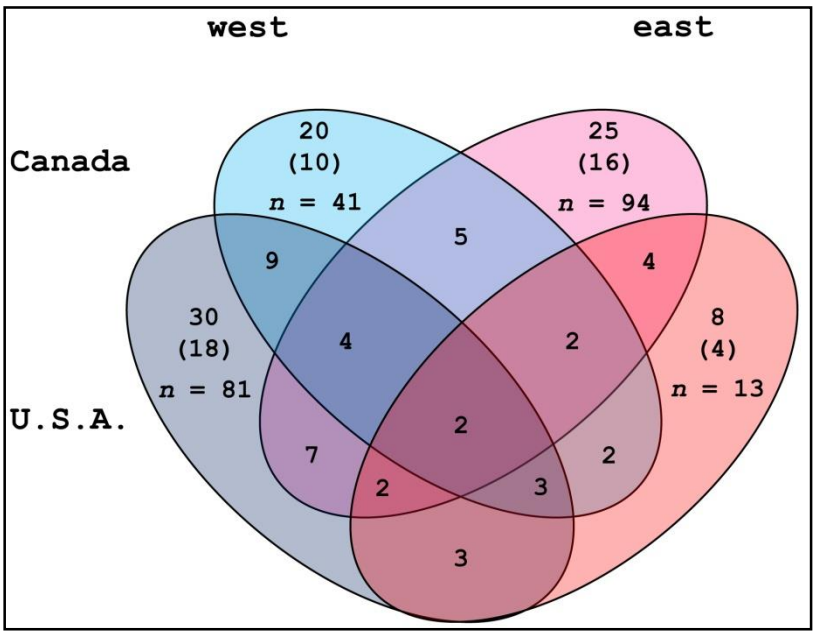


Figure 3.8. Venn diagram representing the number of haplotypes of the mt 12S rRNA + $tRNA^{Val}$ genes found in and shared among key geographical areas. Ovals correspond to: "western" Canada (light blue), "eastern" Canada (pink), "western" U.S.A. (dark blue), and "eastern" U.S.A. (red). The number of haplotypes that were found within a particular geographical region only (in parentheses), as well as the sample size (*n*) corresponding to each region, are also reported. Haplotypes of the *I. scapularis* collected from the nine established populations (*n* = 212) and those of the adventitious ticks (*n* = 17) have been included.

"western" and "eastern" Canada (i.e., five haplotypes) was nearly twice as great as the number in common between "western" and "eastern" U.S.A. (i.e., three haplotypes). Only two haplotypes (i.e., haplotypes CT06 and CT08) were present in all four geographical areas. The number of haplotypes that were unique to (i.e., found only in) "eastern" Canada was 16, while the number of haplotypes that were unique to "western" Canada was only 10. In contrast, the number of haplotypes that were unique to "eastern" U.S.A. was four, while the number of haplotypes that were unique to "western" U.S.A. was 4.5 times as great (i.e., 18 haplotypes).

3.4.2. Concatenated haplotypes of the mt 16S rRNA gene (Is series) and mt 12S rRNA + $tRNA^{Val}$ genes (CT series)

Analysis of the concatenated sequence data showed that there were 98 haplotypes among 228 ticks (Tables 3.7 and 3.8). The most common haplotype was Is-1 + CT08, which comprised 14.5% of the ticks. The second most commonly detected haplotype was haplotype Is-15 + CT07, which comprised 9.2% of the ticks from the different localities. The remaining 96 haplotypes were each represented by 3.1% or fewer of the *I. scapularis*. Most of the haplotypes of the concatenated sequence data were present in one locality only, and most were represented by a single tick.

Tables 3.7 and 3.8 show the number of concatenated haplotypes detected among populations and among adventitious ticks, respectively. Most (i.e., 96.9%) of the haplotypes were detected in only the "western" or only the "eastern" geographical region, but represented 80.3% of the ticks collected. Ninety-seven tick individuals that were collected from different localities within the "western" geographical region comprised 56 haplotypes based on the concatenated dataset, whereas 86 *I. scapularis* individuals that were collected from different localities within the "eastern" geographical region comprised 39 concatenated haplotypes. The remaining tick individuals comprised three haplotypes (i.e., Is-1 + CT06, Is-1 + CT08, and Is-1 + CT25) that were detected in both the "western" and "eastern" geographical regions.

The 40 concatenated haplotypes of 98 ticks that were previously characterized as any of the seven mt 16S rRNA gene haplotypes in Chapter 2 that were detected in both the "eastern" and "western" geographical regions are shown in Table 3.9. Although 92.5% of the haplotypes were detected in only the "western" or only the "eastern" geographical region, these haplotypes represented just 54.1% of the ticks. Thirty-three tick individuals that were collected from the

Table 3.7. The number of *I. scapularis* from the nine established tick populations of each haplotype based on the concatenated data of the mt 16S rRNA gene (Is series) and mt 12S rRNA + $tRNA^{Val}$ genes (CT series). The mt 16S rRNA gene haplotype of each tick was determined in Chapter 2.

Haplotype:		Established population:								
16S 12S	*n*	PVPP	ST	ISP	CR	CSP	PPNP	LPPP	HISK	TPSK
Is-1 CT03	2	0	0	1	1	0	0	0	0	0
Is-1 CT05	3	0	0	0	3	0	0	0	0	0
Is-1 CT06	5	1	0	0	0	2	0	2	0	0
Is-1 CT07	1	0	0	0	0	0	0	1	0	0
Is-1 CT08	33	1	6	3	7	4	7	4	0	1
Is-1 CT09	1	0	0	0	0	1	0	0	0	0
Is-1 CT10	2	0	0	0	0	2	0	0	0	0
Is-1 CT15	1	0	1	0	0	0	0	0	0	0
Is-1 CT19	1	0	1	0	0	0	0	0	0	0
Is-1 CT20	2	0	0	0	0	2	0	0	0	0
Is-1 CT21	1	1	0	0	0	0	0	0	0	0
Is-1 CT25	7	0	0	0	0	1	0	6	0	0
Is-1 CT27	1	0	0	0	0	1	0	0	0	0
Is-1 CT30	3	0	0	0	1	2	0	0	0	0
Is-1 CT32	1	0	0	0	1	0	0	0	0	0
Is-1 CT33	1	1	0	0	0	0	0	0	0	0
Is-1 CT44	1	0	0	0	1	0	0	0	0	0
Is-1 CT45	1	0	0	0	0	0	0	0	0	1
Is-1 CT47	1	0	0	0	0	1	0	0	0	0
Is-1 CT49	1	0	0	0	0	1	0	0	0	0
Is-1 CT50	1	0	0	0	1	0	0	0	0	0
Is-1 CT52	1	0	0	0	0	1	0	0	0	0
Is-1 CT53	1	0	0	0	1	0	0	0	0	0
Is-1 CT54	1	0	0	0	0	0	0	0	1	0
Is-1 CT55	1	0	0	0	0	0	0	1	0	0
Is-1 CT56	2	0	0	0	2	0	0	0	0	0
Is-1 CT57	1	0	0	0	1	0	0	0	0	0
Is-1 CT58	1	1	0	0	0	0	0	0	0	0
Is-1 CT59	1	0	0	0	0	0	0	1	0	0
Is-1 CT61	2	0	0	0	0	0	0	0	2	0
Is-1 CT62	1	0	0	0	0	0	0	1	0	0
Is-2 CT22	1	0	0	0	0	0	1	0	0	0
Is-4 CT29	1	0	1	0	0	0	0	0	0	0
Is-5 CT34	3	0	0	0	0	0	0	0	1	2
Is-6 CT08	1	0	0	0	0	0	0	1	0	0
Is-7 CT40	1	0	0	0	0	0	0	1	0	0
Is-8 CT13	2	0	0	2	0	0	0	0	0	0
Is-9 CT08	1	0	0	1	0	0	0	0	0	0
Is-10 CT01	2	0	0	0	0	0	0	2	0	0
Is-12 CT06	2	0	0	0	0	0	0	0	0	2
Is-13 CT05	1	0	0	0	0	0	0	1	0	0
Is-13 CT08	3	0	0	0	0	0	0	3	0	0
Is-13 CT41	1	0	1	0	0	0	0	0	0	0
Is-14 CT43	3	0	0	2	0	1	0	0	0	0
Is-15 CT06	4	0	0	0	0	0	1	3	0	0
Is-15 CT07	21	0	0	0	0	0	1	20	0	0
Is-17 CT07	1	0	0	0	1	0	0	0	0	0
Is-20 CT14	2	0	0	0	0	0	0	2	0	0
Is-23 CT24	2	0	1	0	1	0	0	0	0	0
Is-30 CT29	1	1	0	0	0	0	0	0	0	0
Is-48 CT08	2	0	0	0	2	0	0	0	0	0
Is-49 CT40	1	0	0	0	0	0	0	0	1	0
Is-50 CT21	2	0	0	0	0	0	2	0	0	0
Is-50 CT23	1	0	0	0	0	0	1	0	0	0

Table 3.7. Continued.

Haplotype:		Established population:								
16S 12S	*n*	PVPP	ST	ISP	CR	CSP	PPNP	LPPP	HISK	TPSK
Is-51 CT23	4	0	3	1	0	0	0	0	0	0
Is-52 CT11	4	0	2	1	0	1	0	0	0	0
Is-53 CT08	2	1	0	1	0	0	0	0	0	0
Is-54 CT07	2	0	0	1	0	1	0	0	0	0
Is-54 CT17	1	1	0	0	0	0	0	0	0	0
Is-55 CT08	1	0	0	0	1	0	0	0	0	0
Is-55 CT27	5	0	0	0	1	4	0	0	0	0
Is-55 CT31	3	0	0	0	3	0	0	0	0	0
Is-56 CT08	2	0	0	0	2	0	0	0	0	0
Is-57 CT28	6	0	5	0	1	0	0	0	0	0
Is-59 CT08	1	0	0	0	0	1	0	0	0	0
Is-59 CT48	1	0	0	0	0	1	0	0	0	0
Is-60 CT08	2	0	0	0	2	0	0	0	0	0
Is-61 CT56	1	0	0	0	1	0	0	0	0	0
Is-62 CT08	4	0	0	0	1	3	0	0	0	0
Is-63 CT07	4	0	0	0	0	0	0	4	0	0
Is-63 CT08	1	0	0	0	0	0	0	1	0	0
Is-64 CT25	1	0	0	0	1	0	0	0	0	0
Is-65 CT45	1	0	0	0	0	1	0	0	0	0
Is-66 CT60	1	0	0	0	0	1	0	0	0	0
Is-67 CT32	1	1	0	0	0	0	0	0	0	0
Is-68 CT07	1	1	0	0	0	0	0	0	0	0
Is-69 CT07	2	2	0	0	0	0	0	0	0	0
Is-69 CT18	1	1	0	0	0	0	0	0	0	0
Is-70 CT29	2	2	0	0	0	0	0	0	0	0
Is-72 CT08	2	0	0	0	0	0	2	0	0	0
Is-73 CT16	2	0	0	0	0	0	0	2	0	0
Is-74 CT02	6	0	0	0	0	0	0	6	0	0
Is-75 CT07	1	0	0	0	0	0	0	1	0	0
Is-76 CT38	3	0	0	0	0	0	0	3	0	0
Is-77 CT39	1	0	0	0	0	0	0	0	1	0
Is-79 CT40	1	0	0	0	0	0	0	0	1	0
Total	211	15	21	13	36	32	15	66	7	6

Table 3.8. The number of adventitious *I. scapularis* individuals of each haplotype based on the concatenated data of the mt 16S rRNA gene (Is series) and mt 12S rRNA + $tRNA^{Val}$ genes (CT series). The mt 16S rRNA gene haplotype of each tick was determined in Chapter 2 or by Krakowetz et al. (2011).

Haplotype:			Region:					
16S	12S	*n*	SK	MB	ON	QC	NB	NS
Is-1	CT45	1	0	0	0	1	0	0
Is-3	CT46	1	0	0	0	1	0	0
Is-4	CT29	1	1	0	0	0	0	0
Is-8	CT12	1	0	1	0	0	0	0
Is-11	CT04	1*	0	0	1	0	0	0
Is-12	CT06	1	0	0	0	0	0	1
Is-15	CT08	1	0	0	1	0	0	0
Is-20	CT37	2	0	0	0	0	1	1
Is-21	CT51	1	0	0	0	1	0	0
Is-24	CT36	1	0	0	0	1	0	0
Is-24	CT42	1	0	0	0	0	0	1
Is-30	CT29	1	0	1	0	0	0	0
Is-58	CT47	1	1	0	0	0	0	0
Is-71	CT26	1	1	0	0	0	0	0
Is-77	CT35	1	0	0	0	0	1	0
Is-78	CT08	1	0	0	1	0	0	0
Total		17	3	2	3	4	2	3

* Tick was collected in 2006 from PPNP (Krakowetz et al. 2011).

Table 3.9. The number of *I. scapularis* from the nine established populations in North America of each haplotype based on the concatenated data of the mt 16S rRNA gene (Is series) and mt 12S rRNA + $tRNA^{Val}$ genes (CT series) that were characterized as any of the seven 16S haplotypes that were detected in both the "eastern" and "western" geographical regions in the study described in Chapter 2. Also included are the haplotype data for two adventitious ticks collected from dogs in Saskatchewan (Is-4 + CT29) and Quebec (Is-1 + CT45).

Haplotype:		Geographical region:	
16S 12S	*n*	Western (includes SK)	Eastern (includes QC)
Is-1 CT03	2	2	0
Is-1 CT05	3	3	0
Is-1 CT06	5	3	2
Is-1 CT07	1	0	1
Is-1 CT08	33	21	12
Is-1 CT09	1	1	0
Is-1 CT10	2	2	0
Is-1 CT15	1	1	0
Is-1 CT19	1	1	0
Is-1 CT20	2	2	0
Is-1 CT21	1	1	0
Is-1 CT25	7	1	6
Is-1 CT27	1	1	0
Is-1 CT30	3	3	0
Is-1 CT32	1	1	0
Is-1 CT33	1	1	0
Is-1 CT44	1	1	0
Is-1 CT45	2	0	2
Is-1 CT47	1	1	0
Is-1 CT49	1	1	0
Is-1 CT50	1	1	0
Is-1 CT52	1	1	0
Is-1 CT53	1	1	0
Is-1 CT54	1	0	1
Is-1 CT55	1	0	1
Is-1 CT56	2	2	0
Is-1 CT57	1	1	0
Is-1 CT58	1	1	0
Is-1 CT59	1	0	1
Is-1 CT61	2	0	2
Is-1 CT62	1	0	1
Is-2 CT22	1	0	1
Is-4 CT29	2	2	0
Is-6 CT08	1	0	1
Is-9 CT08	1	1	0
Is-13 CT05	1	0	1
Is-13 CT08	3	0	3
Is-13 CT41	1	1	0
Is-63 CT07	4	0	4
Is-63 CT08	1	0	1
Total	98	58	40

"western" geographical region comprised 24 haplotypes based on the concatenated dataset, whereas 20 tick individuals that were collected from the "eastern" geographical region comprised 13 concatenated haplotypes. The remaining *I. scapularis* individuals comprised three haplotypes (i.e., haplotypes Is-1 + CT06, Is-1 + CT08, and Is-1 + CT25) that were detected in both the "eastern" and "western" geographical regions.

The haplotype diversity of the *I. scapularis* collected from the nine established populations based on the concatenated sequence data varied from 0.8083 to 0.9810 (Table 3.10). The nucleotide diversity of these ticks ranged from 0.003995 to 0.010804. Chakraborty's test showed that there were significantly more concatenated haplotypes detected than would be expected under neutrality in the population in LPPP. Tajima's and/or Fu's tests revealed no significant negative departure from zero for all but three populations. There were significant differences in the population genetic structure (i.e., F_{ST} values) among most pairs of populations of *I. scapularis* (Table 3.11). The Mantel test showed that there was a statistically significant correlation ($b = 0.000047$, $r^2 = 0.203$, $p = 0.011$) between geographical (km) and genetic (pair-wise F_{ST}) distances (Fig. 3.9).

The Chao 2 curves (not shown) predicted that the total number of concatenated haplotypes in the "western" established populations (i.e., 123 haplotypes) would be fewer than that in the "eastern" established populations (i.e., 147 haplotypes). In contrast, the interpolation and extrapolation curves, despite not reaching their respective asymptotes, showed that the estimated number of haplotypes in the "western" populations was greater than that in the "eastern" populations (Fig. 3.10). Nonetheless, there was no significant difference in estimated haplotype richness based on the rarefaction and extrapolation analyses because of the overlap in 95% confidence intervals of the curves. The rarefaction and extrapolation curves for the combined population data did not reach an asymptote (Fig. 3.11). However, the haplotype diversity was estimated to be 243 based on the results of the Chao 2 estimator. The scatter plot of the total number of haplotypes detected in each population against the total number of ticks collected from each population on the rarefaction curve showed that more haplotypes were detected in the "western" and "eastern" geographical regions than would be expected for a given sample size, except for in ST (MB), LPPP (ON), and possibly PPNP (ON) (Fig. 3.11).

The AMOVA test indicated strong genetic structuring within and among populations in a geographical region (Table 3.12). There was no evidence of genetic structuring among

Table 3.10. Haplotype diversity (h) and nucleotide diversity (π) estimates, and tests of neutrality of the concatenated sequence data (i.e., the mt 16S rRNA gene + mt 12S rRNA + $tRNA^{Val}$ genes) for *I. scapularis* from nine established tick populations in North America.

Site[a]	*n*	No. polymorphic sites	*h*	π	Tajima's test		Fu's test		Chakraborty's test		
					Tajima's D	p^b	*Fs*	p^b	No. haplotypes: Exp.	Obs.	p^b
PVPP	15	17	0.9810	0.005972	-1.37239	0.07800	-6.63746	0.00100	13.22380	13	0.29336
ST	21	17	0.8619	0.003995	-1.00430	0.16000	-1.24829	0.24800	8.78817	9	0.19207
ISP	13	19	0.9359	0.006126	-1.51985	0.06000	-1.69791	0.17800	9.28048	9	0.24502
CR	36	34	0.9508	0.004394	-2.14045	0.00200	-14.18200	0.00000	19.98339	22	0.11080
CSP	32	28	0.9617	0.005156	-1.55411	0.05000	-10.37142	0.00000	20.45952	20	0.15163
PPNP[c]	16	20	0.8083	0.004766	-1.30033	0.08500	-0.62947	0.38300	6.31971	8	0.13792
LPPP	66	33	0.8858	0.006335	-1.50737	0.05500	-3.84870	0.11300	16.21041	21	0.03864
TPSK	6	12	0.8667	0.006715	0.70767	0.74100	1.39614	0.75100	4.30791	4	0.34437
HISK	7	20	0.9524	0.010804	0.15607	0.56700	-0.03367	0.41100	6.07065	6	0.40895

[a] Ticks from MB: PVPP = Pembina Valley Provincial Park and ST = Stanley Trail; MN: ISP = Itasca State Park, CR = Camp Ripley, and CSP = St. Croix State Park; ON: PPNP = Point Pelee National Park and LPPP = Long Point Provincial Park; and South Kingstown, RI: TPSK = Trustom Pond and HISK = Hazard Island.

[b] Significance levels are $p < 0.02$ for Fu's test and $p < 0.05$ for Tajima's and Chakraborty's tests.

[c] Includes the tick collected in 2006 from PPNP (Krakowetz et al. 2011).

Table 3.11. Pair-wise comparisons of geographical (km; upper diagonal) and genetic (F_{ST} values; lower diagonal) distances among nine established populations of *I. scapularis* in North America. Genetic distances (F_{ST}) were determined using the concatenated data of the mt 16S rRNA gene and mt 12S rRNA + $tRNA^{Val}$ genes.

	PVPP[a]	ST	ISP	CR	CSP	PPNP[b]	LPPP	TPSK	HISK
PVPP	--	29	307	426	543	1452	1554	2241	2246
ST	0.08985***	--	326	447	560	1465	1565	2247	2252
ISP	-0.00096	0.06310*	--	123	238	1157	1272	1978	1983
CR	0.08644**	0.00803	0.06059*	--	145	1061	1186	1904	1909
CSP	0.06757***	0.03455***	0.05603*	0.00610	--	921	1042	1758	1763
PPNP	0.02863	0.01475	0.03254	-0.00318	0.01330	--	188	909	914
LPPP	0.06012	0.23738***	0.14125***	0.24279***	0.20871***	0.19107***	--	738	743
TPSK	0.00964	0.17838**	-0.01425	0.17697***	0.09536*	0.10815	0.12778*	--	5
HISK	0.17465*	0.27512***	0.14220**	0.29338***	0.22544***	0.24404**	0.23587***	0.09568	--

*** Significance level $P < 0.001$.
** Significance level $P < 0.01$.
* Significance level $P < 0.05$.
[a] MB: PVPP = Pembina Valley Provincial Park and ST = Stanley Trail; MN: ISP = Itasca State Park, CR = Camp Ripley, and CSP = St. Croix State Park; ON: PPNP = Point Pelee National Park and LPPP = Long Point Provincial Park; and South Kingstown, RI: TPSK = Trustom Pond and HISK = Hazard Island.
[b] Includes the tick collected in 2006 from PPNP (Krakowetz et al. 2011).

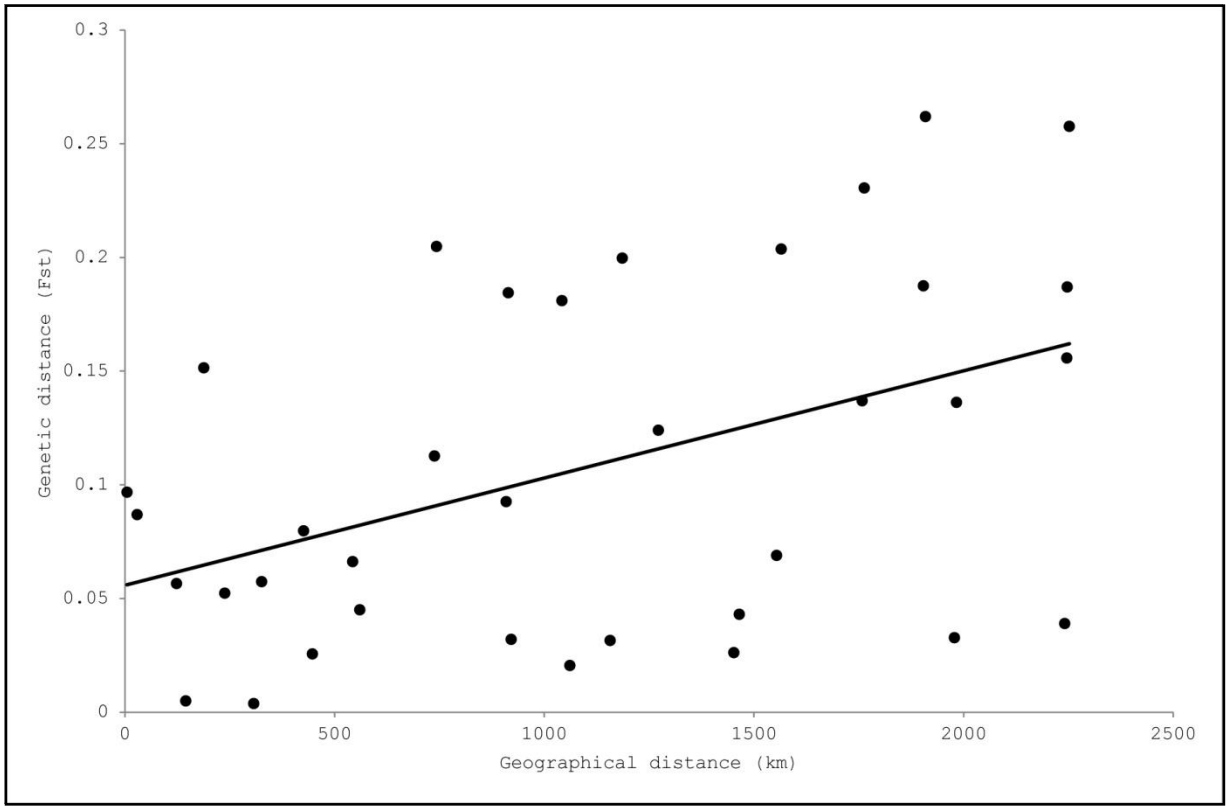


Figure 3.9. Scatter plot derived from the concatenated data depicting the pair-wise comparisons of genetic (F_{ST}) and geographical (km) distances among the nine established populations of *I*. *scapularis* in Canada and the U.S.A.

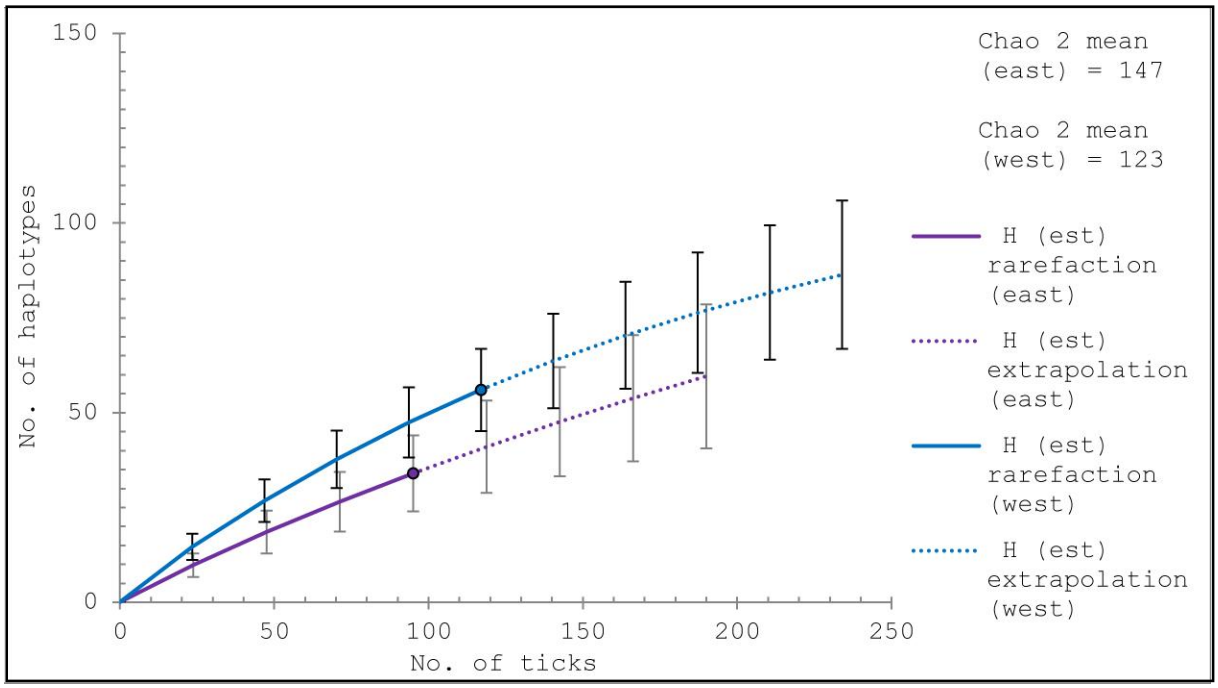


Figure 3.10. Rarefaction and extrapolation curves with 95% confidence intervals based on the concatenated (i.e., mt 16S rRNA gene + mt 12S rRNA + $tRNA^{Val}$ genes) data for the five established populations of *I. scapularis* in the "western" (Pembina Valley Provincial Park, Stanley Trail, Itasca State Park, Camp Ripley, and St. Croix State Park) and for the four in the "eastern" (Point Pelee National Park, Long Point Provincial Park, Trustom Pond, South Kingstown, and Hazard Island, South Kingstown) areas of the U.S.A. and Canada. The total haplotype richness for each of the "western" and "eastern" regions (i.e., 123 and 147 haplotypes, respectively) was estimated using Chao 2 (resulting curve not shown).

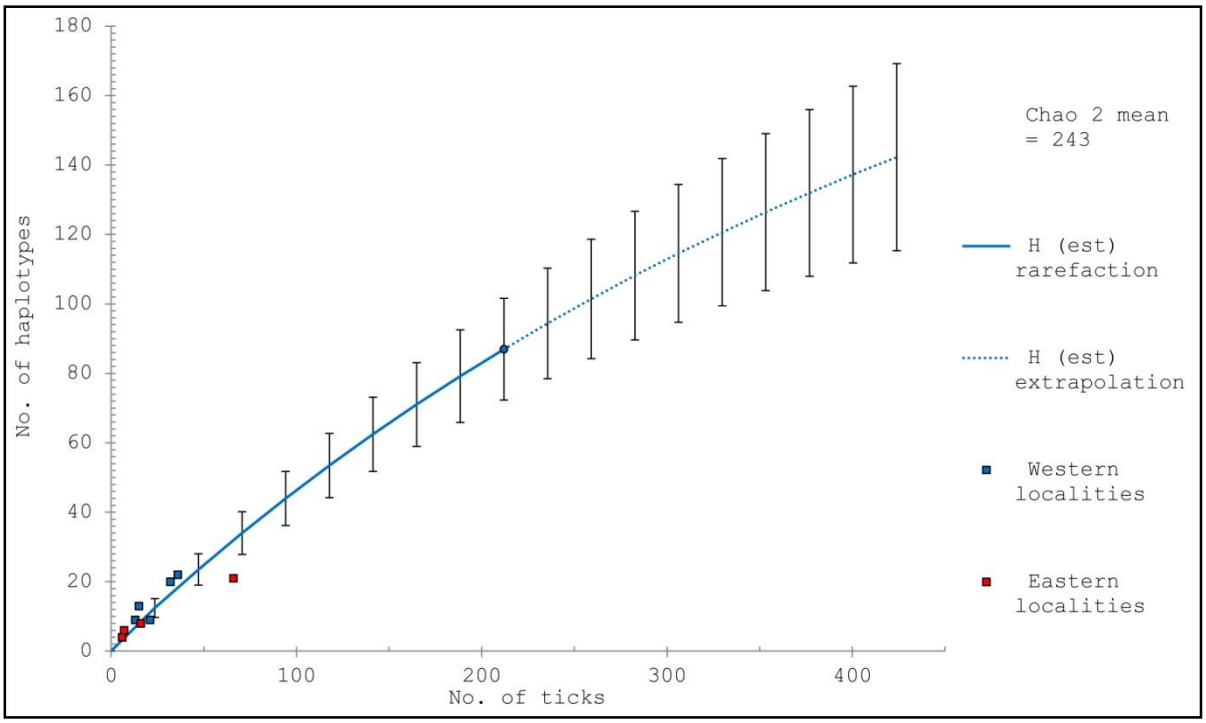


Figure 3.11. Rarefaction and extrapolation curves with 95% confidence intervals based on the concatenated (i.e., mt 16S rRNA gene + mt 12S rRNA + $tRNA^{Val}$ genes) data for the nine established populations of *I. scapularis* in the U.S.A. and Canada, and scatter plot of the number of haplotypes found at each population against the total number of ticks collected from each (see Fig. 3.2 for data). The total haplotype richness for all nine populations (i.e., 243 haplotypes) was estimated using Chao 2 (resulting curve not shown).

Table 3.12. Analysis of Molecular Variance (AMOVA) for nine established populations of *I. scapularis* from Canada and the U.S.A. based on the concatenated data of the mt 16S rRNA gene (Chapter 2) and mt 12S rRNA + $tRNA^{Val}$ genes (present study).

Variance component	*df*	% variance	Fixation index	*p*
Among regions[a]	3	1.7	Φ_{CT} = 0.01687	> 0.05
Among populations within regions	5	4.7	Φ_{SC} = 0.04791	< 0.0001
Within populations	203	93.6	Φ_{ST} = 0.06397	< 0.0001

[a] Regions = Manitoba (PVPP, ST), Ontario (PPNP, LPPP), Minnesota (ISP, CR, CSP) and Rhode Island (TPSK, HISK).

populations of different geographical regions (i.e., provinces of Canada or states of the U.S.A.). Most of the genetic variation (94%) occurred within populations. The phylogenetic analyses using the NJ method on the concatenated dataset were similar to those for the mt 12S rRNA + $tRNA^{Val}$ genes in that there was limited resolution and low bootstrap support for all branches in the tree (not shown).

The minimum spanning network showed that the relationships among the 98 haplotypes was complex, consisting of a mixture of a central haplotype with a radiating "star" pattern and a number of "starburst" patterns (Fig. 3.12). The central haplotype of the "star" pattern (i.e., haplotype Is-1 + CT08) was the most common haplotype and was found in both "eastern" and "western" geographical regions. There was limited separation of haplotypes into "western" and "eastern" groups, except for a single cluster of nine "eastern" haplotypes.

Fig. 3.13 shows the number of haplotypes within the concatenated dataset that were detected in four geographical regions: "western" Canada, "eastern" Canada, "western" U.S.A., and "eastern" U.S.A. In the "western" geographical areas of Canada and the U.S.A., the number of haplotypes detected (i.e., 59 haplotypes; $n = 122$) was nearly 1.5 times greater than that detected in the "eastern" geographical areas (i.e., 42 haplotypes; $n = 106$). The number of haplotypes in common between "western" Canada and "western" U.S.A. (i.e., seven haplotypes) was more than twice as great as the number in common between "eastern" Canada and "eastern" U.S.A. (i.e., three haplotypes). In contrast, the number of haplotypes in common between "western" and "eastern" Canada (i.e., two haplotypes) was similar to the number in common between "western" and "eastern" U.S.A. (i.e., one haplotype). Only one haplotype (i.e., haplotype Is-1 + CT08) was found in all four geographical areas. Ticks collected from localities in southern Canada represented 58 haplotypes ($n = 134$). Similarly, those collected from localities in the northern U.S.A. represented 50 haplotypes ($n = 94$). The number of concatenated haplotypes that were found only in "eastern" Canada was 31, while the number of haplotypes that were unique to "western" Canada was only 17. In contrast, the number of haplotypes that were unique to "eastern" U.S.A. was only six, while the number of haplotypes that were unique to "western" U.S.A. was nearly six times as great (i.e., 35 haplotypes).

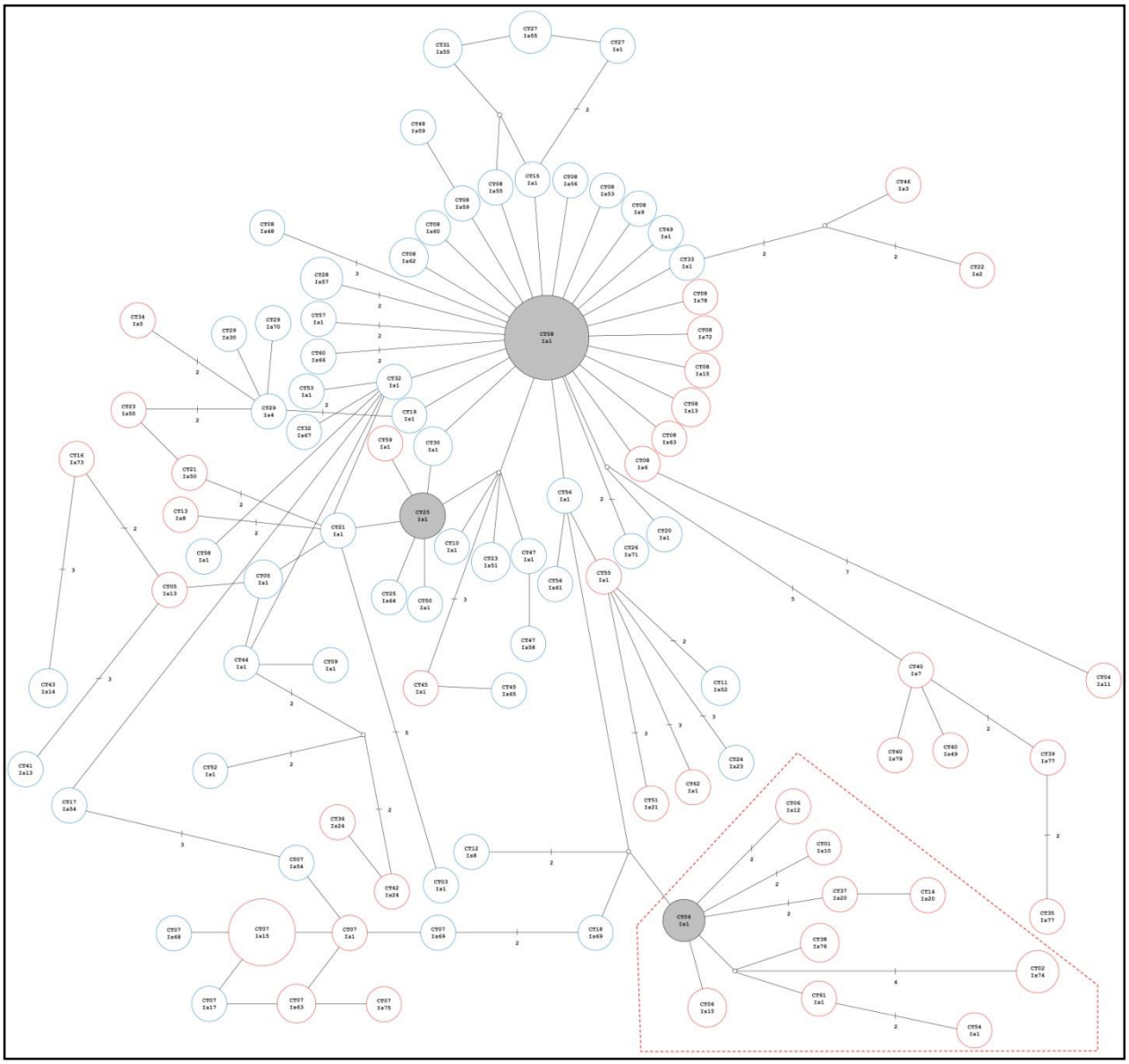


Figure 3.12. Minimum spanning network depicting the relationships among the 98 concatenated haplotypes of the mt 16S rRNA gene (Is series) and the mt 12S rRNA + tNRA^{Val} genes (CT series) of *I*. *scapularis*. The number of nucleotide differences between pairs of DNA sequences (circles) is one unless otherwise indicated. The size of each circle is proportional to the number of ticks of that haplotype. Putative haplotypes are represented by small open circles. Blue, grey, or red circles denote the different haplotypes depending on whether each was detected in the "western," "western" and "eastern," or "eastern" geographical areas, respectively. Contained within the red polygon are haplotypes that were detected in "eastern" geographical regions of Canada and the U.S.A. only, except for haplotype CT06 + Is-1, which was detected in both the "eastern" and "western" geographical regions.

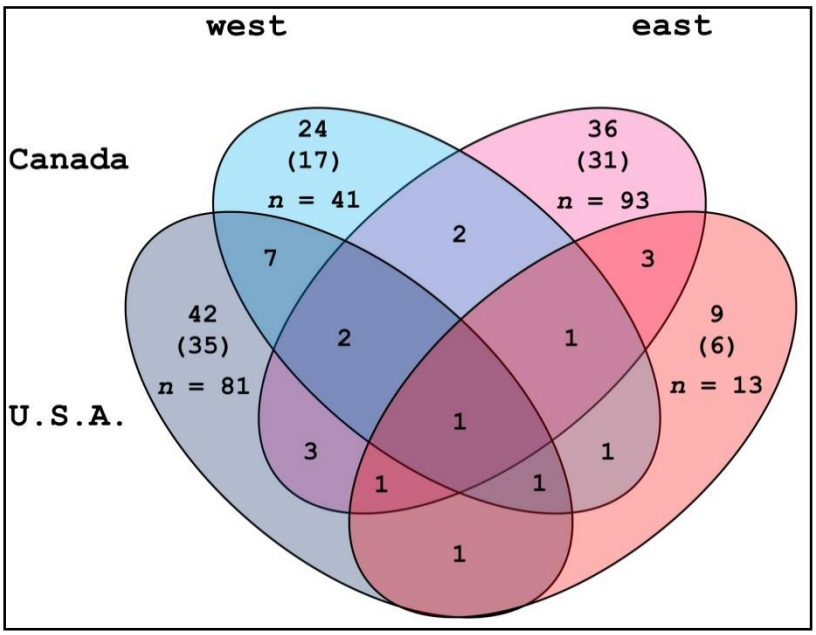


Figure 3.13. Venn diagram representing the number of haplotypes based on the concatenated data found in and shared among key geographical areas. Ovals correspond to: "western" Canada (light blue), "eastern" Canada (pink), "western" U.S.A. (dark blue), and "eastern" U.S.A. (red). The number of haplotypes that were found within a particular geographical region only (in parentheses), as well as the sample size (*n*) corresponding to each region, are also reported. Haplotypes of the *I. scapularis* collected from the nine established populations (*n* = 211) and those of the adventitious ticks (*n* = 17) have been included.

3.5. Discussion

3.5.1. Genetic diversity

Norris et al. (1996) used DNA sequence data of the 3' end of the mt 12S rRNA gene and Domains IV and V of the mt 16S rRNA gene to examine genetic variability in *I. scapularis*. The number of mt 12S rRNA gene haplotypes was not clearly stated in their paper; however, the precise number of haplotypes can be determined by examining the sequence data they deposited in GenBank (accession nos. L43878-L43901). A key finding of their paper was that there were twice as many haplotypes of the mt 12S rRNA gene as compared to the mt 16S rRNA gene, even though their sample size was relatively small (i.e., 24 blacklegged ticks). In the present study, a total of 62 haplotypes of the mt 12S rRNA + $tRNA^{Val}$ genes were detected among the 229 *I. scapularis* examined. As in the study of Norris et al. (1996), more haplotypes of the mt 12S rRNA + $tRNA^{Val}$ genes were detected than of the mt 16S rRNA gene (i.e., 52 haplotypes among 582 blacklegged ticks; Chapter 2). Comparison of the sequence data for the mt 12S rRNA + $tRNA^{Val}$ genes (this chapter) and the mt 16S rRNA gene (Chapter 2) suggests greater genetic variability in *I. scapularis* for the mt 12S rRNA + $tRNA^{Val}$ genes based on higher values for haplotype (0.692-0.933 versus 0.599-0.786, respectively) and nucleotide (0.0050-0.0151 versus 0.0019-0.0036, respectively) diversities. Given these findings, it was anticipated that the mt 12S rRNA gene may more useful than the mt 16S rRNA gene for examining the phylogeography of *I. scapularis* (to be discussed in Section 3.5.3). None of the 62 haplotypes of the mt 12S rRNA + $tRNA^{Val}$ genes detected in this study have been reported previously, as only smaller fragments of the mt 12S rRNA gene have been characterized for *I. scapularis* by others (Norris et al. 1996, Beati and Keirans 2001). Comparisons of each of the sequences of the 62 haplotypes detected in the present study to those available in GenBank have been provided (Table 3.13).

Most of the nucleotide differences in the sequences of the mt 12S rRNA + $tRNA^{Val}$ genes were substitutions. The ratio of transitions to transversions was approximately 1:1, which was consistent with that reported for the mt 12S rRNA gene by Norris et al. (1996). The number of indels (i.e., three) was much lower than that reported by Norris et al. (1996), probably because they included sequence data of haplotypes of the "southern" clade. In general, there was no evidence of directional mutational changes, which was consistent with that reported for the mt 16S rRNA gene (Chapter 2). However, there appeared to be bias among the pyrimidine

Table 3.13. Haplotypes of the mt 12S rRNA + $tRNA^{Val}$ genes for *I. scapularis* individuals in the present study as compared to sequence data (i.e., accession nos.) available for *I. scapularis* on GenBank.

Present study	Chan et al. 2013[a,b]	Beati and Keirans 2001[b]	Norris et al. 1996[b,c]
CT01			
CT02			
CT03			
CT04			
CT05	JX197644[d]		
CT06	JX197475[e]		
CT07	JX197478[f]		
CT08	JX197458[g]	AF150030	L43885
CT09			
CT10			
CT11			
CT12			
CT13	JX197534		
CT14	JX197473		
CT15	JX197458[g]	AF150030	L43885
CT16	JX197644[d]		
CT17			
CT18	JX197475[e]		
CT19	JX197458[g]	AF150030	L43885
CT20			
CT21	JX197536[h]		L43883[i]
CT22			
CT23	JX197458[g]	AF150030	L43885
CT24	JX197469		
CT25	JX197636[j]		
CT26	JX197642[k]		
CT27	JX197559		
CT28			
CT29	JX197458[g]	AF150030	L43885
CT30	JX197636[j]		
CT31			
CT32	JX197536[h]		L43883[i]
CT33	JX197563[l]		
CT34	JX197536[h]		L43883[i]
CT35			
CT36			
CT37	JX197475[e]		
CT38	JX197473		
CT39			
CT40	JX197685[m]		
CT41			
CT42			
CT43	JX197661		
CT44	JX197644[d]		
CT45	JX197561[n]		
CT46	JX197563[l]		
CT47			
CT48			
CT49			
CT50	JX197636[j]		

Table 3.13. Continued.

Present study	Chan et al. 2013[a,b]	Beati and Keirans 2001[b]	Norris et al. 1996[b,c]
CT51	JX197473		
CT52			
CT53			
CT54			
CT55			
CT56	JX197516[o]		
CT57			
CT58	JX197535		
CT59			
CT60	JX197458[g]	AF150030	L43885
CT61			
CT62			

[a] Data available on GenBank only (accession nos. JX197458-JX197781). Last updated 31-JUL-2013. Accessed 1-JAN-2014.
[b] Based on sequences of only ≈307 bp (i.e., missing 128 bp at the 3' end, including part of the mt 12S rRNA gene and the entire $tRNA^{Val}$ gene).
[c] There were no matches between sequences of the present study and those of Norris et al. (1996) based on ≈352 bp (i.e., missing 83 bp at the 3' end, including a small part of the mt 12S rRNA gene and the entire $tRNA^{Val}$ gene).
[d] Also includes JX197645-JX197646, JX197648-JX197659.
[e] Also includes JX197476, JX197479-JX197480, JX197482-JX197484, JX197486-JX197489, JX197494.
[f] Also includes JX197485, JX197490.
[g] Also includes JX197551-JX197554, JX197556-JX197558, JX197560, JX197562, JX197568-JX197573, JX197576-JX197579, JX197581-JX197584, JX197586-JX197587, JX197591, JX197594-JX197599, JX197602-JX197609, JX197612, JX197614-JX197624, JX197630-JX197633, JX197710, JX197713.
[h] Also includes JX197537-JX197550.
[i] Also includes L43884.
[j] Also includes JX197637-JX197640.
[k] Also includes JX197643.
[l] Also includes JX197564-JX197567, JX197580, JX197588, JX197600-JX197601, JX197626.
[m] Also includes JX197686-JX197691, JX197696-JX197698.
[n] Also includes JX197585, JX197589-JX197590, JX197592-JX197593, JX197625, JX197627-JX197629, JX197634.
[o] Also includes JX197517-JX197518, JX197520-JX197522, JX197526, JX197529-JX197533, JX197712.

transitions, as T→C mutational changes were detected nearly four times as often as C→T. It was not clear why this bias exists, as all nine of the pyrimidine transitions occurred at unpaired sites in the secondary structures, and hence did not influence the base pairing in the different helices. Additional studies are needed to determine the significance of this finding.

The locations of the nucleotide differences among haplotypes of the mt 12S rRNA + $tRNA^{Val}$ genes were examined in relation to the secondary structures of these RNAs. The results showed that there was no hypervariable region in these RNAs. In addition, most (90%) of the variable positions in the sequence alignment occurred in unpaired regions, and did not affect the base pairing in the different helices. The remaining variable positions were predominantly comprised of non-compensatory base pair changes, which resulted in the formation of inner bulges on some helical structures. The high number of non-compensatory relative to partial-compensatory base pair changes detected in this study might be a function of its small sample size, as partial-compensatory base pair changes are typically more common than non-compensatory changes and indicate that many mutations at different sequence positions are not occurring independently of one another (Higgs 1998, Li et al. 2008).

The most common haplotype of the mt 12S rRNA + $tRNA^{Val}$ genes was CT08, which was found in four of the eight localities. This haplotype comprised 25% of all ticks examined, which was substantially lower than that reported (i.e., 49%) for the most common haplotype of the mt 16S rRNA gene (i.e., haplotype Is-1) in Chapter 2, which was detected in nine of eleven localities. The next most abundant haplotype (i.e., CT07) comprised 15% of the ticks and was found in only three localities. In contrast, the second most common haplotype of the mt 16S rRNA gene (i.e., haplotype Is-4) represented just 7% of the ticks, but was present in eight localities (Chapter 2). Thus, the pattern of abundance of the different haplotypes differed markedly to that described previously for the mt 16S rRNA gene (Chapter 2).

Analyses were also conducted on the combined sequence data of the mt 12S rRNA + $tRNA^{Val}$ genes (this chapter) and the mt 16S rRNA gene (Chapter 2) for 228 ticks. This resulted in a total of 98 haplotypes. Most of these haplotypes were each represented by a single tick, and, thus, were detected in only one locality. However, two haplotypes were relatively common; Is-1 + CT08 comprised 15% of the ticks, while Is-15 + CT07 comprised 9% of the ticks. The usefulness of the concatenated sequence data for examining the population genetics and phylogeography of *I. scapularis* are discussed below.

3.5.2. Population genetic structure

The number of haplotypes of the mt 12S rRNA + $tRNA^{Val}$ genes detected in each of the nine established populations varied from 4-17. Haplotype CT08 was found in every population, except for HISK, but did not necessarily represent the most frequently detected haplotype in each. For example, in LPPP (ON), the most frequently detected haplotype was CT07, which represented 40% of the ticks from there. Thus, the identities of the haplotypes of the mt 12S rRNA + $tRNA^{Val}$ genes and their proportions differed among the established populations.

The most commonly detected haplotype of the concatenated data was Is-1 + CT08. This observation was not unexpected, as haplotype Is-1 usually represents the most frequently detected haplotype in studies of the mt 16S rRNA gene (Qiu et al. 2002, Humphrey et al. 2010, Krakowetz et al. 2011; Chapter 2), and haplotype CT08 was the most common haplotype of the mt 12S rRNA + $tRNA^{Val}$ genes in the present study. In contrast, the second most common haplotype of the concatenated data (i.e., Is-15 + CT07) was unanticipated, despite haplotype CT07 representing the second most common haplotype of the mt 12S rRNA + $tRNA^{Val}$ genes in the present study, because haplotype Is-15 represented the fourth most commonly detected haplotype in Chapter 2. The high overall prevalence of haplotype Is-15 + CT07 can be explained by the disproportionately large number of ticks of each constituent haplotype in LPPP (ON) relative to the other populations. Specifically, more than 90% of the ticks characterized as Is-15 in the study described in Chapter 2 and nearly 80% of the ticks characterized as CT07 in the present study were collected from LPPP. This suggests that there are differences in the population genetic structure of *I. scapularis* among some of the established populations (e.g., LPPP as compared to each of the other established populations).

There were significant differences in the population genetic structure of *I. scapularis* based on statistical analyses of the F_{ST} data for both data sets between most pairs of established populations, which did not support the hypothesis of gene flow among populations. These results were consistent with those in Chapter 2. The AMOVA tests also did not support the hypothesis of gene flow among populations, as they revealed strong genetic structuring within and among populations in a geographical region, which was consistent with the findings based on the mt 16S rRNA gene (Chapter 2). Additionally, there was no evidence of genetic structuring among populations of different geographical regions, which differed from the results of Chapter 2. This finding may be a function of small sample size. However, the presence of shared haplotypes

among populations supports the hypothesis of gene flow among them. Furthermore, the Mantel tests showed that there were positive and significant correlations between the geographical distances (km) among populations and the magnitude of the genetic differences (i.e., F_{ST} values) among them based on sequences of the mt 12S rRNA + $tRNA^{Val}$ genes and the concatenated data (r^2 = 0.108 and 0.203, respectively). Thus, in general, the greater the geographical distance between two populations, the greater the genetic difference between them.

The observed numbers of haplotypes of the mt 12S rRNA + $tRNA^{Val}$ genes (i.e., 62 haplotypes) and the concatenated data (i.e., 98 haplotypes) within the sampled populations were less than the expected total number of haplotypes, as the Chao 2 estimates were 96 and 243 haplotypes, respectively. This inference was supported by the large number of haplotypes that were each represented by a single tick, as the observed and expected number of haplotypes cannot be considered equal until every haplotype has been sampled two or more times (Colwell and Coddington 1994). It was also supported by the shapes of the interpolation and extrapolation curves, none of which reached an asymptote, suggesting that additional sampling would continue to yield large numbers of previously undetected haplotypes. Furthermore, Chakraborty's test for the mt 12S rRNA + $tRNA^{Val}$ genes revealed that the number of haplotypes detected in the population in CR was greater than the expected number, indicating an excess number of rare haplotypes in that population. For the concatenated data, Chakraborty's test revealed that there were significantly more haplotypes detected than would be expected under neutrality in the population in LPPP. Tajima's and/or Fu's tests for both data sets indicated that there were significant negative departures from zero for some populations, suggesting possible population expansions.

The patterns in the haplotype networks for the mt 12S rRNA + $tRNA^{Val}$ genes and the concatenated data are indicative of a population undergoing expansion (e.g., Qiu et al. 2002, Humphrey et al. 2010). This is consistent with observations that most tick populations in Canada have only recently established or are in the process of establishing. For example, the populations in PVPP and ST in Manitoba have established in the past 1-2 years (L.R. Lindsay 2013, pers. comm., 3 Dec.). However, the population in LPPP in Ontario has been established since the 1970's (Watson and Anderson 1976).

A greater number of mt 12S rRNA + $tRNA^{Val}$ haplotypes were detected in the "western" geographical region than in the "eastern" geographical region (41 and 29 haplotypes,

respectively). The number of shared haplotypes between these regions was eight. Similarly, a greater number of concatenated haplotypes were detected in the "western" than in the "eastern" areas (i.e., 59 and 42 haplotypes, respectively), while the number of shared haplotypes was three. However, there were no significant differences in the total number of estimated haplotypes between the two geographical areas based on the rarefaction and extrapolation curves for both data sets.

3.5.3. Phylogeography

The mt 12S rRNA gene has been used to examine the phylogeographical structure of a Eurasian tick species, *I. persulcatus*, individuals of which were collected from several localities in Russia (Kovalev and Mukhacheva 2012). A total of four haplotypes were detected among the 76 ticks examined, one of which represented 96% of the ticks. These results suggested that the mt 12S rRNA gene may not a suitable marker for investigating the population genetics of *I. persulcatus*. The mt 12S rRNA gene has also been used in combination with the mt control region (CR), the mt cytochrome c oxidase subunit 1 (*cox1*) gene, the mt cytochrome c oxidase subunit 2 (*cox2*) gene, and the mt cytochrome b (*cytb*) gene to examine the intraspecific mt DNA variability in *I. ricinus* (Casati et al. 2008). There was no apparent phylogeographical structure detected for the modest sample size of 26 *I. ricinus* based on the concatenated sequences of the *cox1*, *cox2*, *cytb*, and 12S rRNA genes (Casati et al. 2008). Additional studies have examined the extent of the variability in the mt 12S rRNA gene of different species of tick (Black IV and Piesman 1994, Murrell et al. 1999, Erster et al. 2013, Lu et al. 2013), but not for the purpose of understanding the population genetics or phylogeographical structures of them. Furthermore, it was shown in Chapter 2 that few inferences could be made regarding the phylogeographical relationships of blacklegged ticks based on sequence data of the mt 16S rRNA gene, because 45% of ticks collected from southern Canada were of haplotype Is-1, which also occurred in both the Upper Midwest and Northeast of the U.S.A. Thus, the aim of this study was to determine the extent of the genetic variability in part of the mt 12S rRNA gene of *I. scapularis* and to investigate its usefulness as a population genetics or phylogeographical marker for blacklegged ticks, as the gene has been shown to be somewhat variable within some species of *Ixodes* (e.g., Casati et al. 2008, Kovalev and Mukhacheva 2012) and variable within *I. scapularis* (Norris et al. 1996). Another objective of this chapter was to determine if sequence data of the mt 12S

rRNA + tRNA^{Val} genes combined with data from the mt 16S rRNA gene provides greater resolution of the geographical origins of blacklegged tick populations in Canada.

The sequences of the mt 12S rRNA + tRNA^{Val} genes were determined for 82 blacklegged ticks that were previously characterized as haplotype Is-1 (Chapter 2). The results revealed that haplotype Is-1 comprised 31 haplotypes based on analyses of the 12S rRNA + tRNA^{Val} genes. Forty percent of these ticks were of one haplotype (i.e., CT08). An examination of the geographical distribution of the 31 haplotypes revealed that three haplotypes were found in both the "western" and "eastern" geographical regions, 21 were found in the "western" geographical area only (Prairie Provinces of Canada and Minnesota), while the remaining seven haplotypes were found in the "eastern" geographical area only (Central and Atlantic Provinces of Canada and Rhode Island). Hence, 37 (45%) of the 82 blacklegged ticks characterized previously as haplotype Is-1 were of a haplotype that was found in either the "western" or "eastern" geographical regions, but not both. However, a greater number of ticks of haplotype Is-1 need to be characterized using the 12S rRNA + tRNA^{Val} marker to verify whether these haplotypes are unique to these regions. As 28 of the 31 haplotypes were not shared among the provinces/states of Manitoba, Minnesota, Ontario, or Rhode Island, it was difficult to discern which ticks in Manitoba and Ontario originated from populations in the Upper Midwest (e.g., Minnesota) or the Northeast (e.g., Rhode Island) of the U.S.A. Sample sizes for the subset of ticks from these two Canadian provinces and two states in the U.S.A. ranged from five ticks in Rhode Island to 41 ticks in Minnesota and, thus, were considerably skewed. Therefore, the lack of shared haplotypes among these localities was probably a function of the differing sample sizes. Considering the results of this study and those in Chapter 2, we predict that a more thorough investigation of *I. scapularis* representing haplotype Is-1 would reveal a greater proportion of shared haplotypes between populations in Manitoba and Minnesota and between Ontario and Rhode Island than between Manitoba or Minnesota and Ontario or Rhode Island. However, the outcome of this prediction remains to be determined using larger numbers of ticks.

Differences in the identities of the haplotypes and their proportions among the different localities of southern Canada based on sequences of the mt 12S rRNA + tRNA^{Val} genes in the present study and the concatenated dataset suggest that these ticks may have originated from different geographical regions in the U.S.A. Nine haplotypes based on sequences of the mt 12S rRNA + tRNA^{Val} genes were shared between "western" Canada and "western" U.S.A., which

was greater than the number of haplotypes shared between "eastern" and "western" Canada (i.e., five haplotypes) or between "eastern" and "western" U.S.A. (i.e., three haplotypes). These nine haplotypes represented 45% of the 20 haplotypes detected in "western" Canada and 30% of the 30 haplotypes detected in the "western" U.S.A. The number of haplotypes shared between "eastern" Canada (i.e., ON, QC, NB, and NS) and "eastern" U.S.A. (i.e., RI) was only four, which was greater than the number of haplotypes in common between the "western" and "eastern" geographical regions of the U.S.A., but less than the number of haplotypes in common between the "western" and "eastern" geographical regions of Canada. These four haplotypes represented 16% of the 25 haplotypes detected in "eastern" Canada, but 50% of the eight haplotypes detected in the "eastern" U.S.A. These results support, albeit to a limited extent, the hypothesis that ticks in the Prairie Provinces may have derived from colonizing individuals that originated from established populations in the northern Midwest of the U.S.A., while those in the Atlantic and Central Provinces of Canada may have derived from colonizing individuals that originated from established populations in the Northeast of the U.S.A.

The concatenated data also provided support for the hypothesis of different geographical origins for blacklegged tick populations in southwestern and southeastern Canada. Seven haplotypes were found in both "western" Canada and "western" U.S.A., but only two haplotypes were shared between the "western" and "eastern" regions of Canada. Only one haplotype was shared by the "western" and "eastern" geographical regions of the U.S.A. The seven haplotypes found in both "western" Canada and "western" U.S.A represented 29% of the 24 haplotypes detected in "western" Canada and 17% of the 42 haplotypes detected in the "western" U.S.A. The number of haplotypes shared between "eastern" Canada and "eastern" U.S.A. was three, which was greater than the two shared between "western" and "eastern" Canada and the one shared between the "western" and "eastern" regions of the U.S.A. These three haplotypes represented 8% of the 36 haplotypes found in "eastern" Canada and 33% of the nine haplotypes detected in the "eastern" geographical region of the U.S.A.

The haplotype networks also show associations between some haplotypes and the "eastern" geographical area only, as well as between some haplotypes and the "western" geographical area only. However, these associations are not as unambiguous as those for the mt 16S rRNA gene (Chapter 2).

3.5.4. Conclusions

The DNA sequences of the mt 12S rRNA + $tRNA^{Val}$ genes were more variable than those of the mt 16S rRNA gene. There were significant differences in genetic diversity among established populations of *I. scapularis* based on pair-wise F_{ST} values. There was also evidence of strong genetic structuring based on the AMOVA test within and among populations in a geographical area, with most of the genetic variation detected within populations. However, some haplotypes of the mt 12S rRNA + $rRNA^{Val}$ genes were shared among populations, supporting the hypothesis of gene flow among them. A positive and significant correlation between genetic (F_{ST}) and geographical (km) distances was detected. Another important finding was that large samples sizes are needed to examine the population genetics of *I. scapularis*.

There was an association between some tick haplotypes (both genetic markers) and geographical regions in Canada, which supports the hypothesis of different U.S.A. origins for these ticks. However, there was insufficient resolution based on sequences of the mt 12S rRNA + $tRNA^{Val}$ genes to determine the geographical origins for 27% of the ticks examined from Canada, as these ticks represented one of three haplotypes that were also found in the Upper Midwest and Northeast of the U.S.A. The concatenated data provided greater resolution of the geographical origins of the ticks in southern Canada; however, there was a lack of spatial clustering of haplotypes in the minimum spanning networks, indicating that these genetic markers may not be useful for understanding the patterns of spread of *I. scapularis* on a finer (e.g., regional) scale. Therefore, in the next chapter, the usefulness of the D3 domain and flanking core regions (= $D3^{+}$) of the nuclear large subunit rRNA gene as a genetic marker for population genetic studies of *I. scapularis* is examined. The usefulness of this marker to infer the evolutionary relationships of *I. scapularis* to other species of ixodid ticks is also examined.

CHAPTER 4

AN ASSESSMENT OF GENETIC DIFFERENCES AMONG IXODID TICKS IN A LOCUS WITHIN THE NUCLEAR LARGE SUBUNIT RIBOSOMAL RNA GENE[3]

4.1. Abstract

We examined the usefulness of the D3 domain and flanking core regions (= $D3^+$) of the nuclear large subunit (LSU) ribosomal DNA as a genetic marker for species level identification and the inference of evolutionary relationships of ixodid ticks. Genetic variation was also examined in relation to the secondary structure of the LSU rRNA. The results revealed a lack of sequence difference in the $D3^+$ among species of *Dermacentor* and among some species of *Ixodes*, demonstrating that this gene region is not suitable as a species marker for all species of ixodid ticks. Of the 45 variable nucleotide positions in the sequence alignment of the $D3^+$, 23 did not alter the secondary structure of the LSU rRNA, because they occurred in unpaired positions, whereas 16 represented partial- or full-compensatory changes which maintained the secondary structure. Six deletions in the $D3^+$ sequence of all *Ixodes* species examined resulted in a shorter d4_1 helix compared with that of other tick species. The phylogenetic analyses also showed that the $D3^+$ is of limited value in resolving evolutionary relationships among ixodid ticks. In addition, we demonstrated that the $D3^+$ of ascomycete fungi could be amplified along with, or instead of, the $D3^+$ of some tick species, depending upon the primers used in PCR. Nonetheless, the $D3^+$ of the fungal contaminants are readily distinguished from the $D3^+$ of ixodid ticks because of a shorter length and the absence of helix d4_1 in the secondary structure of the LSU rRNA.

4.2. Introduction

Ticks are important vectors of human and animal pathogenic agents (e.g., viruses, bacteria, and protozoa) in different regions of the world (Estrada-Peña and Jongejan 1999). The accurate identification of individual ticks to the species level is an important requirement for the

[3] Part of this chapter was reproduced with permission from Elsevier (http://www.elsevier.com/about/policies/author-agreement/lightbox_scholarly-purposes): **Anstead CA, Krakowetz CN, Mann AS, Sim KA, Chilton NB (2011)** An assessment of genetic differences among ixodid ticks in a locus within the nuclear large subunit ribosomal RNA gene. Mol Cell Probes 25: 243-248. NBC conceived the project and organized the collection of samples. CAA collected some samples. CAA, CNK, ASM, and KAS carried out laboratory work. CAA, CNK, and NBC performed the data analyses and interpreted the data. All authors wrote the manuscript and approved the final manuscript. CAA and CNK contributed equally to the manuscript.

establishment of effective programs aimed at controlling and managing tick populations and for the treatment of diseases caused by tick-borne pathogens. However, it is sometimes difficult to unequivocally identify ticks at all life cycle stages to species because of morphological similarities among closely related species (Andrews et al. 1992, Jackson et al. 2000, Anderson et al. 2004, Andrews et al. 2006). This is, particularly, the case for immature stages (larvae and nymphs) and adult females having fed on hosts, and for ticks that are damaged following their removal from a host (Andrews et al. 1992, Jackson et al. 2000, Anderson et al. 2004, Andrews et al. 2006). Therefore, the geographical localities from which ticks are collected are sometimes used to aid in their identification, but this approach can be problematic when morphologically similar species occur in sympatry (Jackson et al. 2000). As a consequence, biochemical and molecular techniques have been developed to identify ticks (Andrews et al. 1992, Poucher et al. 1999, Jackson et al. 2000, Anderson et al. 2004, Andrews et al. 2006).

Several nuclear and mitochondrial DNA genes provide useful genetic markers for the identification of individual ticks to the species level, irrespective of life cycle stage or their state of engorgement (Poucher et al. 1999, Anderson et al. 2004, Guglielmone et al. 2006, Dergousoff and Chilton 2007, Mtambo et al. 2007). These markers have also been used to study the population genetics of ticks and to infer their evolutionary relationships (Black IV and Piesman 1994, Klompen et al. 2000, Qiu et al. 2002, Guglielmone et al. 2006, Dergousoff and Chilton 2007, Krakowetz et al. 2010). The D3 (divergent) domain or expansion segment of the nuclear large subunit (LSU) ribosomal RNA gene has been used as a marker for phylogenetic studies on a variety of arthropods, including chelicerates (Wheeler and Hayashi 1998), mites (Maraun et al. 2004), and ticks (McLain et al. 2001). The D3 domain has also been used to compare blacklegged ticks (*Ixodes scapularis*) from different parts of their distributional range and as a species marker for some oribatid mites (Maraun et al. 2003). Previous studies of ixodid ticks (McLain 2001, McLain et al. 2001, Maraun et al. 2003) showed that there were marked differences in the D3 sequences of six species of *Ixodes*, suggesting that this DNA region is a suitable species marker and that it would be useful for phylogenetic studies on ticks. In the present study, we explored the nature and extent of the sequence variation within and among several species of ixodid tick representing the two major subfamilies, the Prostriata and Metastriata. Nucleotide alterations in the DNA sequences of the D3 domain and flanking regions of the nuclear LSU rRNA gene (= $D3^{+}$) were also examined in relation to the ribosomal RNA

secondary structure. Furthermore, we assessed the utility of this region of the LSU to infer the phylogenetic relationships of ixodid ticks.

4.3. Materials and methods

Total genomic DNA (gDNA) was extracted and purified from 131 individual ticks, representing 10 species (*Ixodes angustus*, *n* = 8; *Ixodes kingi*, *n* = 12; *Ixodes ricinus*, *n* = 4; *I. scapularis*, *n* = 78; *Ixodes sculptus*, *n* = 12; *Dermacentor andersoni*, *n* = 4; *Dermacentor albipictus*, *n* = 4; *Dermacentor variabilis*, *n* = 7; *Dermacentor occidentalis*, *n* = 1; and *Rhipicephalus sanguineus*, *n* = 1), using the methods described previously (Dergousoff and Chilton 2007). The $D3^+$ was amplified from gDNA by PCR using the forward (5'-GTGAAT TCACCCGTCTTGAAACAC-3') and reverse (5'-GTGGATCCTGAGGGAAACTTCGG-3') primers of McLain et al. (2001). Reactions were performed in 25 µl volumes containing 1-2 µl of gDNA, 250 µM of each dNTP (Bio-Rad, Bio-Rad Laboratories Inc., Hercules, California, U.S.A.), 3.5 mM $MgCl_2$ (Bio-Rad), 25 pmol of each primer, and 0.5 U of i*Taq*™ DNA polymerase (Bio-Rad). The PCR conditions used were 95°C for 5 min, then 30 cycles of 95°C for 30 sec, 48°C for 30 sec and 74°C for 30 sec, followed by 74°C for 5 min. No template (i.e., negative) controls were included with each set of PCR reactions. Amplicons were compared on 1.5% agarose-TBE gels (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3; OmniPur®, EMD Millipore Ltd., Etobicoke, Ontario (ON), Canada) and stained using SYBR® Safe DNA Gel Stain (Life Technologies Inc., Burlington, ON, Canada). In addition, single-strand conformation polymorphism (SSCP) analyses were conducted to screen for genetic variation within species. SSCP was performed using the materials and methods described previously (Gasser et al. 2006, Dergousoff and Chilton 2007, Krakowetz et al. 2010).

Representative amplicons were either purified using spin columns (MinElute® PCR Purification Kit, Qiagen, Toronto, ON, Canada) or ExoSAP-IT (Affymetrix, MJS BioLynx Inc., Brockville, ON, Canada), and subjected to automated DNA sequencing using the forward and reverse primers in separate reactions. All DNA sequences were compared with sequence data in GenBank™ (using BLASTn®). Sequences were aligned manually. Variable positions in the $D3^+$ sequence alignment were examined in relation to the secondary structure of the LSU rRNA based on the model of Wuyts et al. (2001). Phylogenetic analyses were conducted on the sequence data using the neighbour-joining (NJ) and maximum parsimony (MP) methods in

PAUP (Swofford 2002). The D3$^+$ sequence of the holothyroid mite, *Allothyrus* cf. *constrictus* (GenBank accession no. AY626629), was used as the outgroup in the analyses. For the MP analysis, gaps were treated as a fifth character state, and all character states were assigned an equal value. The consistency index (CI), excluding uninformative characters, and the retention index (RI) were recorded for the most parsimonious trees.

4.4. Results and discussion

The D3$^+$ amplicons produced from the majority of tick gDNA samples were ~380 bp in size on agarose gels; however, smaller-sized amplicons (~330 bp) were produced from the gDNA of 6 *I. sculptus* and 7 *I. kingi* (Fig. 4.1A). In addition, the amplicons of three *I. sculptus* and two *I. kingi* individuals had two bands (~330 and 380 bp) on an agarose gel (Fig. 4.1A). The sequences of the 380 bp amplicons from the *I. sculptus* and *I. kingi* gDNA were 99% similar to the LSU rDNA of a related species, *Ixodes cookei* (GenBank accession no. AY62631; Klompen et al. 2007). In contrast, a BLASTn search of the sequences of the 330 bp amplicons from *I. sculptus* and *I. kingi* revealed that they were identical to the LSU sequences of ascomycete fungi (GenBank accession nos. AB470555 and FJ567949, respectively). Amplification of the LSU rDNA of fungal contaminants from invertebrate gDNA samples is often a problem, given the relatively high genetic similarity in the sequences and secondary structure for many regions of the LSU rRNA gene among distantly related organisms, particularly in the core regions of the rDNA (Wuyts et al. 2001). The sequence of the regions flanking the D3 domain of the ascomycete fungus associated with *I. kingi* was 85% similar (i.e., 28 bp differences over 183 alignment positions) to that of *I. kingi*, but only 49% similar for the D3 domain (i.e., 82 bp differences over 160 alignment positions). There were 61 point mutations (37 transitions and 24 transversions) and 49 indels when comparing the D3$^+$ sequences of *I. kingi* and its associated fungus. The relatively shorter D3 domains of the two fungal species compared with those of *I. kingi* and *I. sculptus* were evident based on the absence of stem d4_1 and a reduced d5 stem (Fig. 4.2).

As a consequence of the fungal contamination in some amplicons, two new primers, namely Tick-28S-C2-F (5'-GCGGCGAGTAGGTCGGTAACC-3') and Tick-d9-D3-R (5'-ACGTCAGAATCGCTTCGGA-3'), were designed to amplify the D3$^+$ of ticks (and other arthropods), but not the D3$^+$ of fungi. These primers were tested using the same PCR conditions

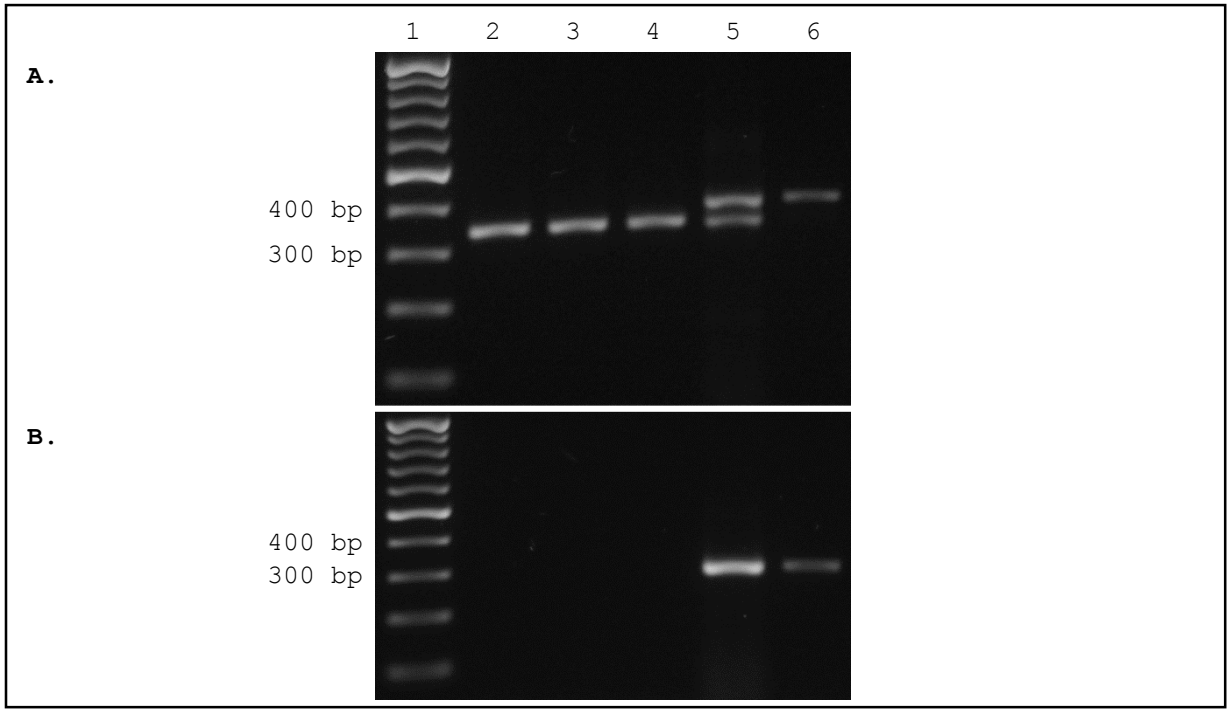


Figure 4.1. (A) An agarose gel displaying the amplicons produced by PCR from gDNA of individual *I. kingi* (lanes 2 to 6) using primers reported by McLain et al. (2001). Amplicons of ~380 bp and ~330 bp are those of the $D3^+$ LSU of ticks and fungi, respectively. (B) Agarose gel of the amplicons produced by PCR of the same gDNA samples, but using primers Tick-28S-C2-F and Tick-d9-D3-R (designed herein). Amplicons of ~300 bp are those of the $D3^+$ LSU of ticks, and not fungi. A standard DNA ladder (GeneRuler™ 100 bp Plus DNA Ladder 100 to 3000 bp, Thermo Fisher Scientific, Ottawa, ON, Canada) was used as a size standard on both gels (lane 1).

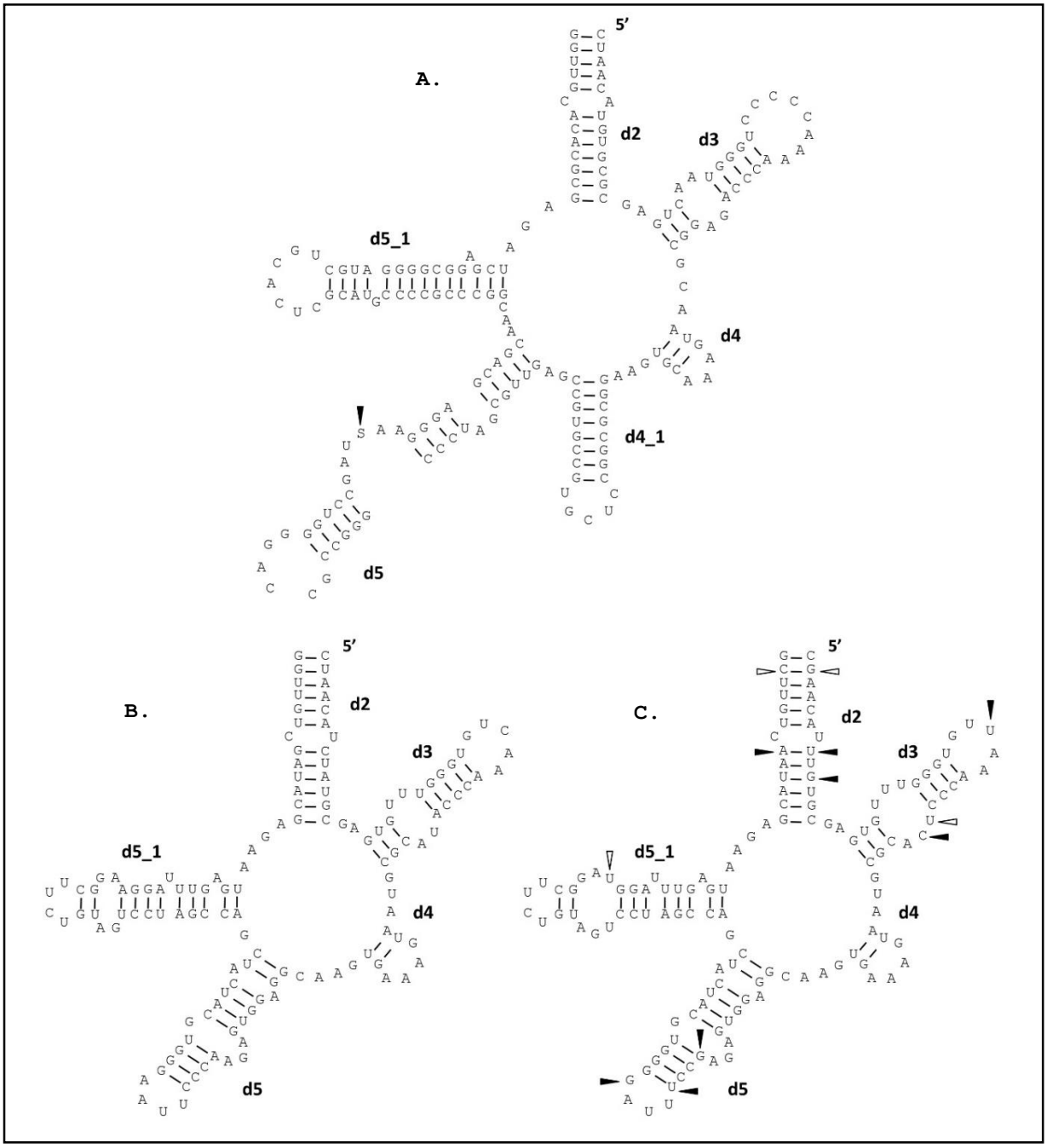


Figure 4.2. The secondary structures of the D3 region of the LSU rRNA gene for (A) *I. kingi* and *I. sculptus* (solid arrow indicating the interspecific difference), and the ascomycete fungi associated with gDNA samples of (B) *I. kingi* and (C) *I. sculptus*. Helices are numbered (d2 to d5_1) according to the model of Wuyts and co-workers (2001). Closed and open arrows on the secondary structure of the *I. sculptus* associated fungus indicate the transitional and transversional sequence differences (respectively) compared with the *I. kingi* associated fungus.

as specified above, except that the annealing temperature was raised to 60 °C. No amplicons were produced for the fungal contaminants present in the tick gDNA (Fig. 4.1B), whereas the positive amplicons were confirmed to be the D3$^+$ of *I. kingi* and *I. sculptus* by DNA sequencing.

There was no evidence of amplification of fungal LSU rDNA from the gDNA of the other tick species, following amplification with forward and reverse primers described by McLain et al. (2001) (or with primers Tick-28S-C2-F and Tick-d9-D3-R) based on amplicon size and BLASTn searches of the sequence data produced. For *Dermacentor*, no intraspecific variation was detected in the D3$^+$ sequences of *D. variabilis* collected from geographically isolated populations in Saskatchewan, Canada (n = 4) and California, U.S.A. (n = 3), or among multiple individuals of *D. albipictus* and *D. andersoni*. In addition, there were no interspecific differences in D3$^+$ sequences among representative individuals of the four species of *Dermacentor* (Table 4.1). The sequences of these ticks were also identical to that for a *Dermacentor* (indeterminate species) collected from a dog in Ewartsville, Washington, U.S.A. (GenBank accession no. AY859582; Mallatt and Giribet 2006). The D3$^+$ sequence determined for a single adult of *R. sanguineus* was identical to that of the published sequence for *R. sanguineus* (GenBank accession no. AF062986; Wheeler and Hayashi 1998), except for two transitional changes (i.e., C's compared to Y and T in the sequence with accession no. AF062986 at nucleotide positions 111 and 343; Fig. 4.2).

The D3$^+$ sequences of all 5 *Ixodes* species (340 bp) determined in the present study were 4 bp longer than those of *R. sanguineus* and the 4 *Dermacentor* species (i.e., 336 bp). SSCP profiles for 78 *I. scapularis* adults collected from Manitoba and Nova Scotia in Canada (n = 1 and 3, respectively) and Minnesota in U.S.A. (n = 74) were the same (not shown), but they differed from those of other *Ixodes* species (Fig. 4.3). The lack of variation in the SSCP profiles among *I. scapularis* adults inferred an absence of intraspecific variation in the D3$^+$ sequence. This inference was supported by the DNA sequencing results for 5 representative *I. scapularis* adults. The D3$^+$ sequences of these ticks were identical to one another (340 bp), but differed from the D3$^+$ sequences of the 4 *I. ricinus* individuals at a single nucleotide position (i.e., alignment position 88) and at 4-10 nucleotide positions when compared with the other *Ixodes* species (Table 4.1). Except for *I. angustus* and *I. sculptus*, which were identical in sequence to one another, all *Ixodes* species examined had a different D3$^+$ sequence. The present sequence results

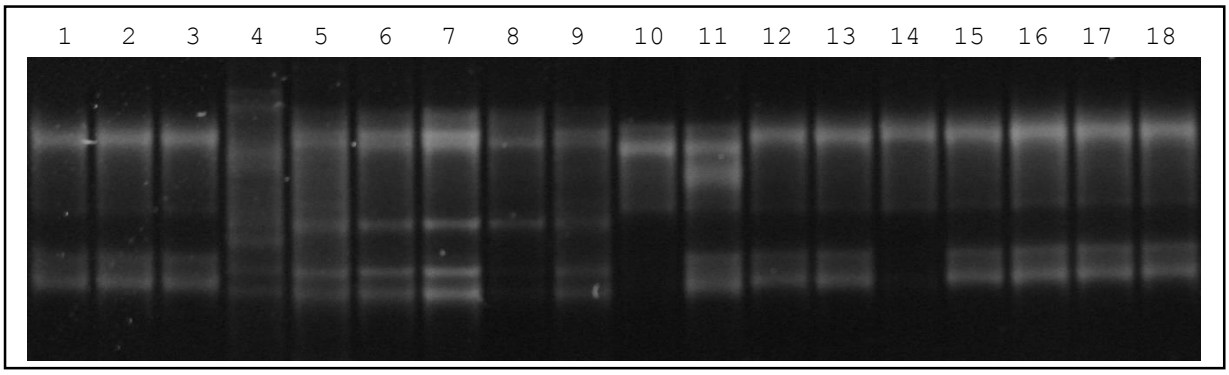


Figure 4.3. Single-strand conformation polymorphism (SSCP) profiles of the $D3^{+}$ LSU rDNA for individual adults of *I*. *angustus* (lanes 1-3 and 15-18), *I*. *ricinus* (lane 4), *I*. *scapularis* (lanes 5-9), *I*. *kingi* (lanes 10 and 11), and *I*. *sculptus* (lanes 12-14).

Table 4.1. Variable nucleotide positions in the aligned D3+ sequences of 12 species of ixodid tick (Family Ixodidae). A dot indicates the same nucleotide as in the sequence of *I. kingi*. Nucleotide positions 25-184 are located in the D3 domain, while positions 1-24 and 185-344 are located in the flanking regions (see Fig. 4.4).

| Species | Alignment position: | | |

for representatives of two genera, *Ixodes* and *Dermacentor*, show that the D3 region is not a suitable genetic marker for the specific identification of all ixodid ticks to the species level.

The magnitude of sequence differences in the D3 domain (i.e., 0-5%, excluding flanking regions) among the five species of *Ixodes* examined herein (i.e., *I. angustus*, *I. kingi*, *I. ricinus*, *I. scapularis*, and *I. sculptus*) was significantly less than the 11-32% sequence differences reported previously in studies of *Ixodes* (McLain 2001, McLain et al. 2001), which also included *I. scapularis* and *I. ricinus*. The sequences determined for *I. scapularis* and *I. ricinus* also differed significantly (i.e., at 15% and 11% of 192 and 188 alignment positions, respectively) to the published sequences for these species (GenBank accession nos. AF303987 and AF303988, respectively; McLain 2001, McLain et al. 2001). Moreover, the D3 and flanking sequences of *I. scapularis* and *I. ricinus* from McLain et al. (2001) differed from one another at 30 of 192 (16%) alignment positions, whereas only a single nucleotide difference was detected between these two species (Table 4.1). Given the differences in sequence results between studies, two internal primers (forward: 5'-TGAGGCGAATGAAACGCC-3' and reverse: 5'-TCTAGCTAG CTCACGTCG-3') were designed based on the published sequence for *I. scapularis* (i.e., GenBank accession no. AF303987) by McLain et al. (2001). However, no amplicons were detected on agarose gels following PCR of the gDNA of *I. scapularis* adults using these internal primers together or in combination with forward and reverse primers described by McLain et al. (2001). In addition, BLASTn searches of the previously published D3 sequences of *I. scapularis* and *I. ricinus* (McLain et al. 2001) showed very little genetic similarity to the LSU sequences of any arachnid, whereas the $D3^{+}$ sequences determined for these two tick species were 81-87% similar to those of a variety of arachnid species. As a consequence of these findings, we did not include any sequences from McLain et al. (2001) in our determination of the nucleotide alterations in the secondary structure of the tick $D3^{+}$ rRNA (Fig. 4.4) or in the phylogenetic analyses (Fig. 4.5).

The results of the present study revealed that the magnitude of sequence differences in $D3^{+}$ among tick species within a genus (i.e., 0% for *Dermacentor* and 0-3% for *Ixodes*) was less than that among genera (2-11%) (Table 4.1). This finding is consistent with the reported genetic differences in the D3 region among oribatid mites of the same genus (0-6%; Maraun et al. 2003) and the more substantial sequence differences among genera of oribatid mites (Maraun et al. 2004). There were 45 variable nucleotide positions in the $D3^{+}$ among tick species, 39 of which

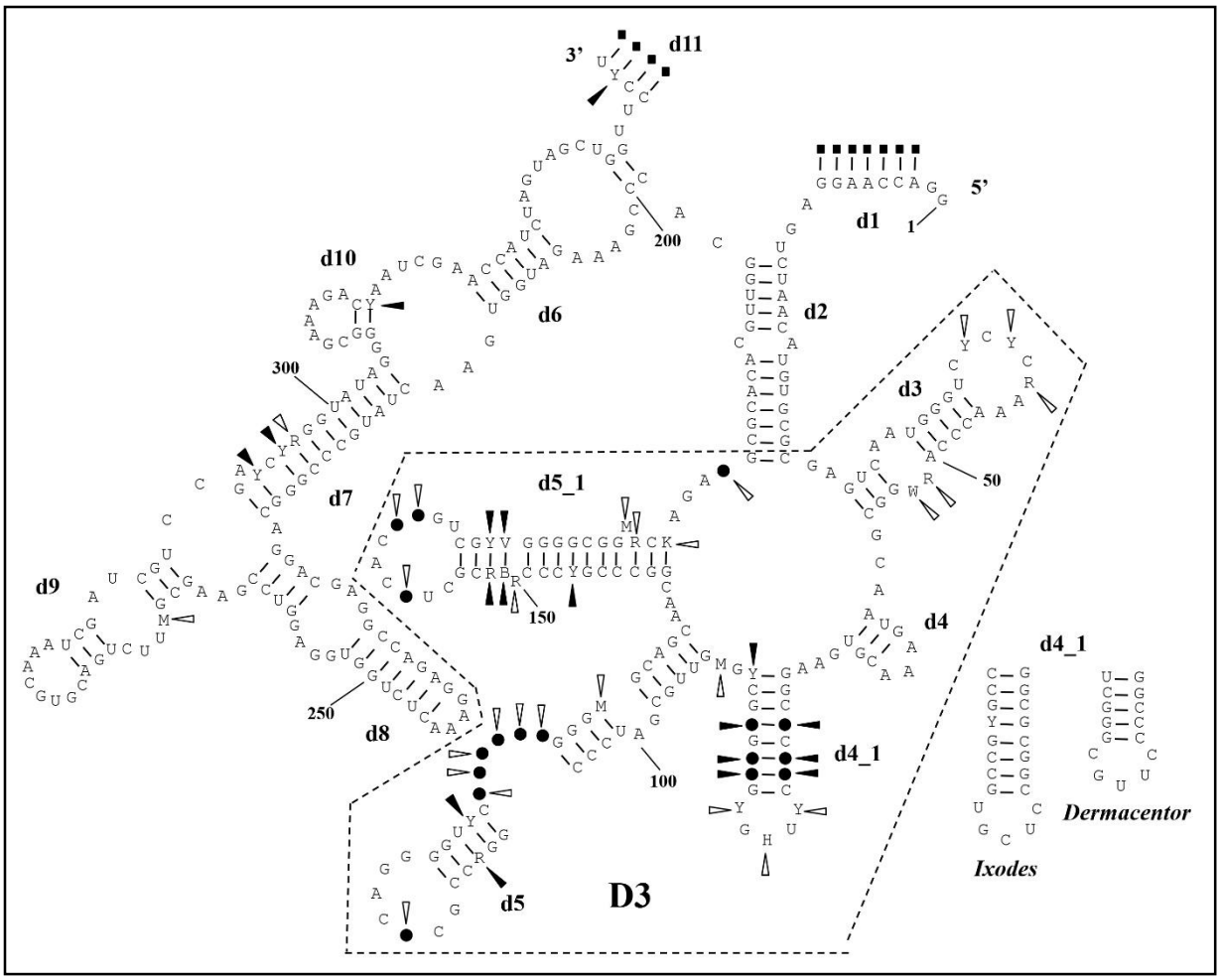


Figure 4.4. Variable nucleotide positions in the D3 domain and flanking regions ($D3^{+}$) of the LSU rRNA gene for 12 species of ixodid tick (see Table 4.1). Solid arrows indicate partial or complete compensatory nucleotide alterations that maintain base pairing of stems. Open arrows indicate nucleotide alterations of unpaired positions (e.g., loops and bulges) or partial compensatory nucleotide alterations that do not maintain the base pairing on stems. Solid circles represent indels, while solid squares represent nucleotide positions from other regions of the LSU rDNA that are involved in base pairing with the $D3^{+}$ (Wheeler et al. 1998). Helices are numbered (d1-d11) according to the model of Wuyts and co-workers (2001).

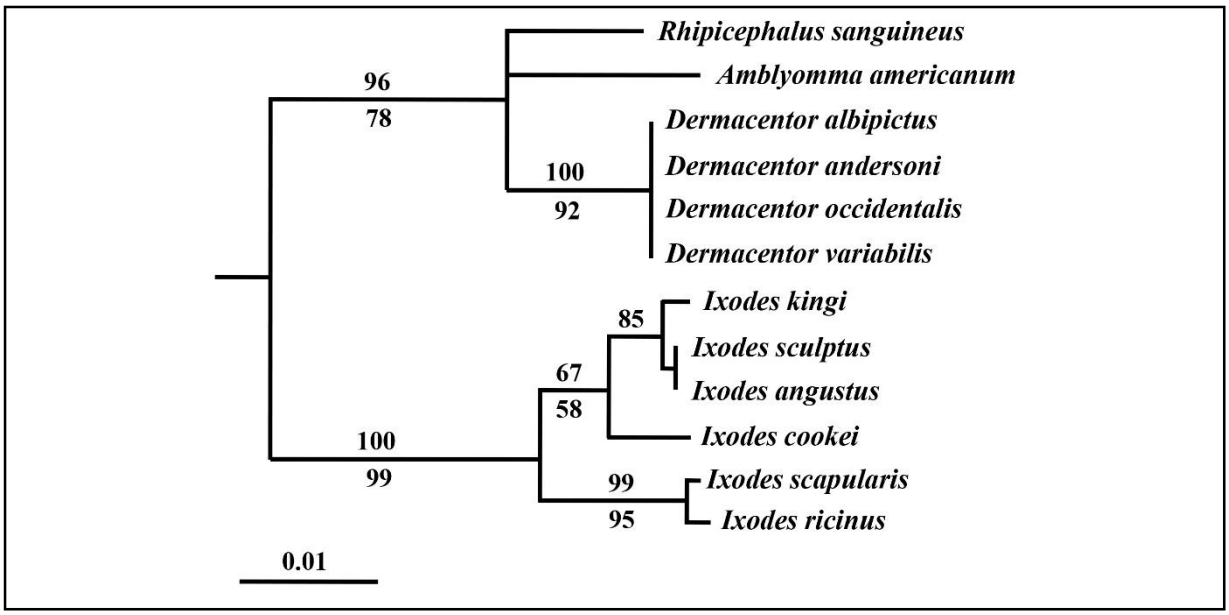


Figure 4.5. Phylogenetic relationships of the 12 species of ixodid tick inferred from a neighbour-joining (NJ) analysis of sequence data of the $D3^{+}$ of the LSU rRNA gene. Values above and below branches are the bootstrap support (based on 1,000 replications) for NJ and MP analyses, respectively. Sequence data of the mite *Allothyrus* cf. *constrictus* was used as the outgroup for the NJ and MP analyses.

were detected in the D3 domain. The nucleotide differences represent 25 point mutations (19 transitions and 6 transversions), 11 indels, and 9 multiple changes (i.e., indel, transition, and/or transversion). With respect to the secondary structure of the $D3^+$ rRNA (Fig. 4.4), approximately half (i.e., 23) of the nucleotide alterations occurred in unpaired positions on stems or in the end loops of stems. Eight transitional changes (positions 91, 107, 120, 146, 249, 296, 316, and 343) represented partial-compensatory changes which maintained the secondary structure. Nucleotide alterations at four positions (151, 152, 167, and 168) represented complementary changes on both sides of helix d5_1. The six indels (positions 74, 76, 77, 85, 86, and 88) in the sequence of all *Ixodes* species represented full-compensatory base pair changes on helix d4_1, resulting in a longer helix for this part of the D3 domain compared with the other tick species (Fig. 4.4).

The phylogenetic tree produced from the NJ analyses of the $D3^+$ sequences (Fig. 4.5) resulted in the separation of the 12 tick species into two major groups (clades): one containing the 6 species of *Ixodes* and the other including representatives of the genera *Amblyomma*, *Dermacentor*, and *Rhipicephalus*. There was very strong statistical support (i.e., bootstrap values of 96-100%) for each of these clades. Within the genus *Ixodes*, there was strong support (bootstrap value: 99%) for a sister taxon relationship between *I. scapularis* and *I. ricinus*, and for a clade (bootstrap value: 85%) that included *I. kingi*, *I. sculptus*, and *I. angustus*. Of the 45 variable positions in the $D3^+$ sequences of the 12 ixodid tick species (Table 4.1), 38 were informative in the maximum parsimony (MP) analysis. These analyses produced two equally most parsimonious trees (L = 100, CI = 0.81, and RI = 0.91) (not shown). As in the NJ analyses, there was strong bootstrap support for monophyly of species within the genus *Ixodes*, and for a sister taxon relationship between *I. scapularis* and *I. ricinus*. Both *I. scapularis* and *I. ricinus* belong to the subgenus *Ixodes*, whereas the other four *Ixodes* species belong to different subgenera (*Ixodiopsis* and *Pholeoixodes*). As in the NJ tree, there was no resolution of the relationships among species of the other genera (i.e., *Dermacentor*, *Amblyomma*, and *Rhipicephalus*) in the consensus MP tree. The placement of *Ixodes* into a different clade from the other three genera is consistent with the separation of the Ixodidae into the subfamilies Prostriata and Metastriata, respectively (Hoogstraal and Aeschlimann 1982), and the findings of other molecular studies that have examined the evolutionary relationships of ixodid ticks (Black IV and Piesman 1994, Klompen et al. 2000).

In conclusion, the $D3^{+}$ region of the LSU rDNA is not suitable as a species marker for all species of ixodid ticks because of a lack of sequence differences among some species of *Ixodes*, and among the four species of *Dermacentor* examined in the present study. However, this gene region is of some use for examining the evolutionary relationships of different genera of ixodid ticks.

CHAPTER 5

CHARACTERIZATION OF THE DNA SEQUENCE AND SECONDARY STRUCTURE OF THE COMPLETE MITOCHONDRIAL 16S RIBOSOMAL RNA GENE OF *IXODES SCAPULARIS*[4]

5.1. Abstract

The complete DNA sequence and secondary structure of the mitochondrial (mt) 16S ribosomal (r) RNA gene were determined for six *Ixodes scapularis* adults. The DNA sequences varied in length from 1,240-1,244 bp, but were similar in A+T composition (~82%). The 44 variable positions in the sequence alignment consisted of five purine transitions, seven pyrimidine transitions, 13 transversions, and 19 indels. Most (95%) of these mutational changes did not affect the integrity of the secondary structure of the mt 16S rRNA, because they either occurred in unpaired positions or represented partial- or full-compensatory changes that maintained base pairing in the helices. The results also showed that there are several regions of the mt 16S rRNA gene that may provide appropriate sites for population genetics and phylogeographical studies of *I. scapularis*.

5.2. Introduction

Several nuclear and mitochondrial (mt) DNA regions have been used to examine the population genetics and phylogeographical relationships of *Ixodes scapularis* (Norris et al. 1996, Qiu et al. 2002, Trout et al. 2009, Humphrey et al. 2010, Krakowetz et al. 2011, Mechai et al. 2013, Van Zee et al. 2013). The marker most often used in these studies is an ~400 bp fragment (i.e., Domains IV and V) of the mt 16S ribosomal (r) RNA gene (Norris et al. 1996, Qiu et al. 2002, Trout et al. 2009, Humphrey et al. 2010, Krakowetz et al. 2011). It was shown in Chapter 2 that this part of the gene was highly variable in DNA sequence among ticks collected from southern Canada and northern parts of the U.S.A. Furthermore, there was support for the hypothesis that populations of *I. scapularis* that occur in different regions of southern Canada

[4] Part of this chapter was reproduced with permission from Elsevier (http://www.elsevier.com/about/policies/author-agreement/lightbox_scholarly-purposes): **Krakowetz CN, Chilton NB (2015)** Sequence and secondary structure of the mitochondrial 16S ribosomal RNA gene of *Ixodes scapularis*. Mol Cell Probes 29: 35-38. NBC conceived the project and organized the collection of samples. CNK carried out laboratory work. CNK and NBC performed the data analyses, interpreted the data, wrote the manuscript, and approved the final manuscript.

originated from colonising individuals derived from different populations in the U.S.A. However, few inferences could be made regarding the phylogeographical relationships of *I. scapularis* in Chapter 2, because 45% of ticks collected from southern Canada were of a haplotype (i.e., Is-1) that was common in both the Upper Midwest and Northeast of the U.S.A. Furthermore, it was shown that another ~430 bp region of the mt genome encompassing the 3' end of the 12S rRNA gene and the $tRNA^{Val}$ gene also lacked sufficient resolution to determine the geographical origins of the blacklegged ticks in southern Canada, because 27% of the *I. scapularis* collected from Canada comprised one of three haplotypes (i.e., CT06, CT08, and CT45) that were also found in the Upper Midwest and Northeast of the U.S.A. Concatenation of the sequence data for the mt 16S and 12S rRNA + $tRNA^{Val}$ genes provided greater clarity as to the potential U.S.A. origins of *I. scapularis* in southern Canada; however, it did not completely resolve the geographical origins of the Canadian ticks. Therefore, other genetic markers need to be used in combination with Domains IV and V of the mt 16S rRNA gene (Chapter 2) and the mt 12S rRNA + $tRNA^{Val}$ genes (Chapter 3) to provide greater resolution of the geographical origins of ticks in southern Canada. In this chapter, the complete mt 16S rRNA gene was characterized for a small number of *I. scapularis* individuals to determine if other domains of this gene may be useful for examining the population genetics of *I. scapularis* and/or for inferring the phylogeographical relationships of the established populations of this socio-economically important haematophagous arthropod.

5.3. Materials and methods

Total genomic (g) DNA was extracted and purified from six *I. scapularis* (see Krakowetz et al. 2011; Chapter 2). Each tick was previously characterized using ~400 bp of the 3' end of the mt 16S rRNA gene, as described by Krakowetz et al. (2011) and in Chapter 2. The ticks represented five different mt 16S rRNA gene haplotypes: haplotype Is-1 (tick nos. IS-F-1 and CS-F-13), haplotype Is-9 (tick no. IS-F-4), haplotype Is-11 (tick no. PP06-2-10), haplotype Is-12 (tick no. BH06-2-1), and haplotype Is-17 (tick no. CR-F-22) (Krakowetz et al. 2011; Chapter 2). The complete mt 16S rRNA gene was amplified from the gDNA of each tick by PCR using the primers Tick-12S-1 (5'-AAACTAGGATTAGATACCC-3') and Tick-ND1-F2 (5'-AGGAAG CTTAAATTCCT-3'), which were designed based on sequence alignments of the mtDNA sequences of *Ixodes ricinus* (GenBank™ accession no. JN248424) and *Ixodes persulcatus*

(GenBank accession no. AB073725). Both tick species, together with *I. scapularis*, are members of the subgenus *Ixodes* (Xu et al. 2003). PCRs were performed in 25 µl volumes containing a 1X final concentration of *Taq* Buffer with KCl (Fermentas, Fisher Scientific, Ottawa, Ontario (ON), Canada), 2 mM $MgCl_2$ (Fermentas), 200 µM of each dNTP (Fermentas), 1 uM of each primer, 0.5 U of *Taq* DNA Polymerase (Fermentas), and 1 µl of gDNA template. A negative control containing no gDNA was included in the run. The cycling conditions used for the PCRs were 95ºC for 5 min, then 35 cycles of 94ºC for 1 min, 53ºC for 1 min, and 72ºC for 1 min, followed by a final 5 min extension at 72ºC. Amplicons were compared by electrophoresis at 140 V for 50 min on a 1.5% (w/v) agarose-TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3; OmniPur®, EMD Millipore Ltd., Etobicoke, ON, Canada) gel that was stained using SYBR® Safe DNA Gel Stain (Life Technologies Inc., Burlington, ON, Canada). Amplicons (~1,800 bp) were then purified using the methodology described in Chapter 2 and subjected to automated DNA sequencing using the primers Tick-12S-1 and Tick-ND1-F2 in separate reactions. Sequences were aligned manually, but modified according to the predicted secondary structure of the mt 16S rRNA that was constructed for *I. scapularis* based on the secondary structure models of this gene for other eukaryotes (Gutell and Fox 1988, Gutell et al. 1993, Gutell 1996). Variable positions in the DNA sequence alignment were examined in relation to the predicted secondary structure. Contingency tests (χ^2) were used to test if there were significant differences between the proportion of variable positions between the 5' and 3' halves of the 16S rRNA gene. The TCS program (Clement et al. 2000) was used to generate a minimum spanning network tree to depict the relationships of the different sequence haplotypes.

5.4. Results

Amplicons derived from each tick represented a single band of the expected size (i.e., ~1,800 bp) based on the sequences of *I. persulcatus* and *I. ricinus* on an agarose gel (not shown), whereas no band was detected in the negative control sample (also not shown). The DNA sequences of the complete mt 16S rRNA gene for the six ticks varied in length from 1,240 to 1,244 bp, and their A+T contents were ~82%. Each sequence was unique over the entire gene for each tick and differed from the other sequences by 10-25 bp (i.e., 0.8-2.0%) (Table 5.1). The relationships among the six haplotypes are shown in Fig. 5.1. There was no central haplotype in

Table 5.1. The variable nucleotide positions in the complete mitochondrial 16S rRNA gene sequences of six individuals of *I. scapularis*. A dot (.) at an alignment position indicates the same nucleotide as in the sequence of tick no. IS-F-1, while a dash (–) represents a gap.

| Tick no.[a] | Alignment position: | | |

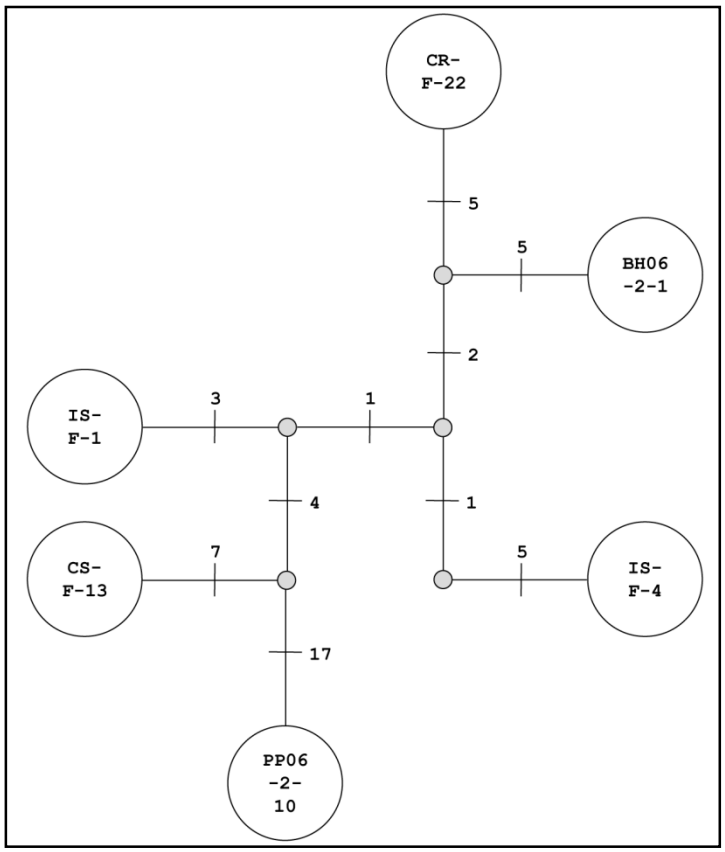


Figure 5.1. A minimum spanning network tree depicting the relationships among the DNA sequences of the complete mt 16S rRNA gene of six *I. scapularis*. The white circles represent the six sequences (or haplotypes), whereas the grey circles represent hypothetical intermediates or unsampled haplotypes. The number of nucleotide differences between pairs of haplotypes is indicated between each pair.

the minimum spanning network tree, and, thus, no "star" pattern. Instead, a multi-branched pattern is exhibited among the haplotypes.

There were 44 variable positions in the 1,252 bp alignment of the sequences. These comprised 25 substitutions in approximately a 1:1 ratio of transitional to transversional mutations (i.e., 12 and 13, respectively) and 19 indels (i.e., insertions/deletions) (Table 5.1). Furthermore, the number of purine transitional changes (i.e., five) was approximately equal to the number of pyrimidine transitional changes (i.e., seven). The predicted secondary structure of the complete mt 16S rRNA is shown in Fig. 5.2. Of the 44 variable positions in the sequence alignment, 25 occurred in the first half (i.e., Domains I and II = alignment positions 1-577) of the mt 16S rRNA gene, while the remaining 19 occurred in the second half (i.e., Domains IV to VI = alignment positions 578-1,252) of the gene. There was no significant difference in the number of variable positions between the two halves of the mt 16S rRNA gene ($\chi^2_1 = 2.11$, $p > 0.05$).

Thirty-three (i.e., 75%) of the variable positions in the sequence alignment occurred in unpaired regions (i.e., end loops or bulges of helices) and, thus, did not alter the pairing arrangements of helices in the secondary structure (Fig. 5.2). Three purine transitional changes at positions 789, 956, and 1,214 in the alignment each represented a partial-compensatory base pair change (i.e., a mutation on one side of a helix that is associated with the maintenance of base pairing), while, together, two transversional changes at positions 118 and 138 in the alignment represented a full-compensatory base pair change (i.e., complementary mutations, one on each side of a helix, that are associated with the maintenance of base pairing). Four indels in the DNA sequence of one tick (i.e., BH06-2-1) at positions 510, 511, 541, and 542 in the sequence alignment (Table 5.1) represented a loss of two base pairs in one helix of the secondary structure (Fig. 5.2). There was a single transversional change in the sequence of another tick (i.e., no. CR-F-22) at position 183 in the alignment (Table 5.1) that represented a non-compensatory mutational change resulting in the formation of an inner bulge on a helical structure (Fig. 5.2). In the sequence of tick no. CS-F-13 at position 255 in the alignment (Table 5.1), a second non-compensatory mutational change resulted in the formation of an inner bulge on a helical structure (Fig. 5.2).

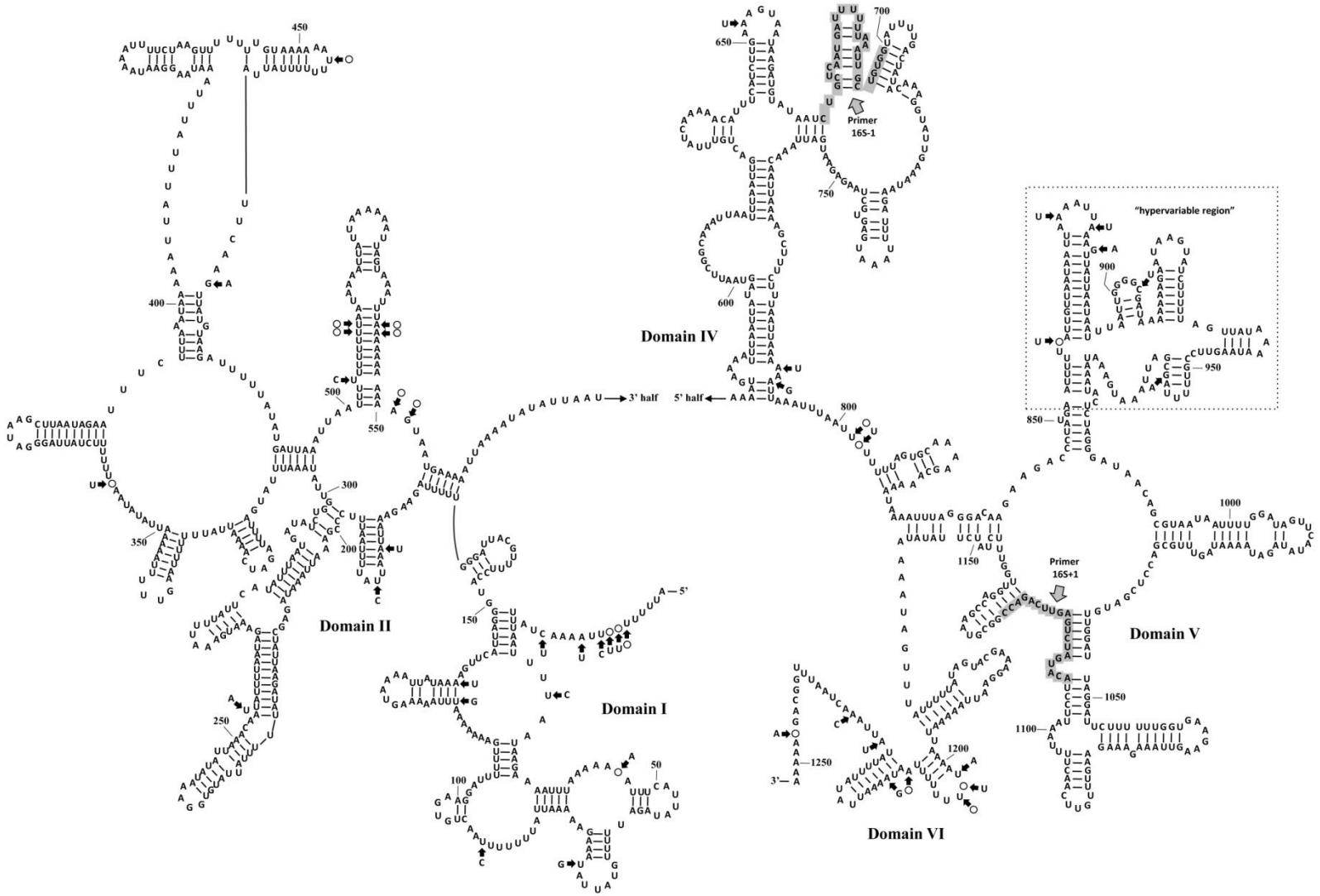


Figure 5.2. The secondary structure of the complete mt 16S rRNA gene for *I. scapularis* according to the models of Gutell and Fox (1988), Gutell et al. (1993), and Gutell (1996). Black arrows indicate mutations relative to specimen IS-F-1 in the 1,252 bp alignment of the six sequences. The box represents the "hypervariable region" (e.g., Black and Piesman 1994; Misof et al. 2000).

5.5. Discussion

The DNA sequences of the complete mt 16S rRNA gene of *I. scapularis* (1,240-1,244 bp) were similar in length to that of *I. ricinus* (1,250 bp; Montagna et al. 2012), but ~30 bp longer than those of *Ixodes uriae* (1,210 bp), *Ixodes holocyclus* (1,214 bp), and *I. persulcatus* (1,206 bp) (Shao et al. 2005a) and ~40 bp shorter than that of *Ixodes hexagonus* (1,287 bp; Black IV and Roehrdanz 1998). The A+T contents of these sequences (82%) were also similar to those of other species of *Ixodes*: *I. hexagonus* (73%), *I. uriae* (78%), *I. persulcatus* (80%), *I. ricinus* (81%), and *I. holocyclus* (82%) (Black IV and Roehrdanz 1998, Shao et al. 2005a, Dermauw et al. 2009, Montagna et al. 2012). A high A+T content in the DNA sequences of the mt 16S rRNA gene has also been reported in many other arthropods (Black IV and Roehrdanz 1998, Lavrov et al. 2000, Navajas et al. 2002, Mitani et al. 2004, Shao et al. 2004, Shao et al. 2005a, Shao et al. 2005b, Shao et al. 2006, Jeyaprakash and Hoy 2007, Domes et al. 2008, Van Leeuwen et al. 2008, Dermauw et al. 2009). The predicted secondary structure of the mt 16S rRNA for *I. scapularis* was similar to that of other arthropods (Gutell and Fox 1988, Misof and Fleck 2003, Gillespie et al. 2006, Domes et al. 2008, Li et al. 2013) in that there were five domains (i.e., Domains I, II, and IV-VI) rather than six, as there are in the secondary structures of many other eukaryotes (Gutell and Fox 1988, Gutell et al. 1993, Gutell 1996, Lydeard et al. 2000).

Each of the six *I. scapularis* examined had a unique sequence of the complete mt 16S rRNA gene, even though two individuals (i.e., tick nos. IS-F-1 and CS-F-13) were previously characterized as haplotype Is-1 using ~400 bp (i.e., Domains IV and V) of this gene (Krakowetz et al. 2011; Chapter 2). The number of differences in DNA sequence among the six blacklegged ticks for the complete mt 16S rRNA gene ranged from 10-25 bp, whereas the number of differences among these six ticks in Domains IV and V of the gene (i.e., between primers 16S-1 and 16S+1 in the alignment of the complete gene; Fig. 5.2) varied from 0-7 bp. The pattern of the minimum spanning network tree depicted in Fig. 5.1 was not the "star" pattern that was observed among haplotypes of the "American" clade based on sequences of *I. scapularis* in Domains IV and V of this gene (Krakowetz et al. 2011; Chapter 2), but rather a multi-branched pattern.

Most (~95%) of the variable positions in the aligned sequences had no effect on or maintained the integrity of the secondary structure of the ribosome, because the mutational changes in DNA sequence either occurred in unpaired regions (i.e., end loops or bulges of

helices) or represented partial- or full-compensatory base pair changes (i.e., mutations on one or both sides of a helix that are associated with the maintenance of base pairing). This is consistent with the findings of other studies that compared the secondary structure of the mt 16S rRNA in a variety of eukaryotes, including insects, crustaceans, arachnids, molluscs, and lizards (Machado et al. 1993, Buckley et al. 2000, Lydeard et al. 2000, Misof et al. 2002, Misof and Fleck 2003, Smith and Bond 2003, Brown 2005, Sorokina et al. 2005). The 44 variable positions in the sequence alignment were equally distributed across the two halves of the mt 16S rRNA gene. Similarly, there was no significant difference in the number of variable sites that represented indels in Domains I and II (i.e., 12 indels) as compared to the number in Domains IV-VI (i.e., seven indels). Investigation of the mutational changes in Domains I, II, and VI of the mt 16S rRNA gene detected among the six DNA sequences revealed there are other suitable sites for population genetics and phylogeographical studies of *I. scapularis*, in addition to those most commonly used (i.e., those in the ~400 bp of Domains IV and V; positions 700-1,106; Fig. 5.2).

In the present study, the six blacklegged ticks were selected for investigation because they represented ticks from the "eastern" and "western" geographical regions of the U.S.A. and Canada, as defined in Chapter 2. More importantly, two ticks (i.e., tick nos. IS-F-1 and CS-F-13) were specifically selected for comparison, because they represented the same haplotype (i.e., haplotype Is-1) based on sequence analyses of the ~400 bp region of Domains IV and V of the mt 16S rRNA gene (Chapter 2). Haplotype Is-1 is the most common haplotype of the "American" clade in the Upper Midwest and Northeast of the U.S.A., and represents the majority of ticks found within the established populations in southern Canada (Rich et al. 1995, Qiu et al. 2002, Humphrey et al. 2010, Krakowetz et al. 2011, Van Zee et al. 2013; Chapter 2). Thus, the U.S.A. origins of ticks of haplotype Is-1 (and several other haplotypes) in southern Canada could not be determined. Comparison of the two DNA sequences across the complete mt 16S rRNA gene for ticks IS-F-1 and CS-F-13 revealed differences between them at 14 alignment positions; however, most (i.e., 79%) of these mutational differences occurred in Domains I and II. Therefore, it is likely that many other ticks characterized as haplotype Is-1 can be further differentiated based on comparative sequence analyses of Domains I and II, rather than comparing sequences of Domains IV, V, or VI. It may also be possible to use DNA sequence data from Domains I and II to distinguish individuals of other common haplotypes based on Domains IV and V that occur in blacklegged tick populations both in the Midwest and Northeast

of the U.S.A., and, thus, further resolve the geographical origins of *I. scapularis* in southern Canada.

In conclusion, determination of the geographical origins in the U.S.A. of the different populations of *I. scapularis* in Canada will provide a better understanding of our risk of exposure to pathogenic bacteria (e.g., *Borrelia burgdorferi* or *Anaplasma phagocytophilum*), because some strains and the prevalences of some strains of pathogenic bacteria differ between the Upper Midwest and Northeast of the U.S.A. (Massung et al. 2002, de la Fuente et al. 2005, Gatewood et al. 2009, Hoen et al. 2009, Brisson et al. 2010, Margos et al. 2012). The results of this chapter suggest that the 5' end of the mt 16S rRNA gene may represent a useful genetic marker for determining the geographical origins in the U.S.A. of *I. scapularis* in southern Canada. This is examined in the next chapter.

CHAPTER 6
GENETIC VARIATION IN DOMAINS I AND II OF THE MITOCHONDRIAL 16S RIBOSOMAL RNA GENE OF THE BLACKLEGGED TICK, *IXODES SCAPULARIS*

6.1. Abstract

Genetic diversity in the 5' end (i.e., Domains I and II) of the mitochondrial (mt) 16S ribosomal (r) RNA gene of 577 *Ixodes scapularis* individuals was examined. Mutational differences were located at 74 positions in the sequence alignment, and a total of 130 sequence variants (i.e., haplotypes) were detected. Interpolation and extrapolation curves were constructed based on the sequence data and showed that many more haplotypes remain to be found. There were shared haplotypes among geographical areas, supporting the hypothesis of gene flow among them. Sequence data of the 3' end (i.e., Domains IV and V) of the mt 16S rRNA gene (Chapter 2) were combined with these data for each tick (n = 576). Additionally, sequence data of the 3' end of the mt 12S rRNA gene and the complete transfer (t) RNA^{Val} gene (Chapter 3) were concatenated with the combined data of the 5' and 3' ends of the mt 16S rRNA gene for 225 ticks. Haplotype networks were constructed for each dataset: 1) the 5' end of the mt 16S rRNA gene (~300 bp), 2) the 5' and 3' ends of the mt 16S rRNA gene (~700 bp), and 3) the mt 12S rRNA + $tRNA^{Val}$ genes + the 5' and 3' ends of the mt 16S rRNA gene (~1130 bp), and each network revealed a shallow genealogical structure for *I. scapularis*. There was some evidence of associations between some tick haplotypes (all genetic markers) and geographical areas in Canada, but, in general, there was a lack of spatial clustering of haplotypes in the haplotype networks. These results are consistent with the life history of the species in northeastern North America in terms of its dispersal patterns as facilitated by migratory passerines.

6.2. Introduction

Phylogeography is a field of study in which phylogenetic relationships among individuals within a species and among closely related species are investigated in light of their historical and contemporary geographical distributions (Avise 2000). The goal of the field is to better understand the principles and processes that have affected the geographic distributions of genealogical lineages (Avise 2000). In Chapters 2 and 3, the population genetics and phylogeography of *I. scapularis* were examined using Domains IV and V of the mt 16S rRNA

gene and a DNA fragment consisting of the 3' end of the mt 12S rRNA gene and the entire $tRNA^{Val}$ gene, respectively, in order to determine the geographical origins in the U.S.A. for the ticks collected in Canada. The determination of the U.S.A. origins of the Canadian ticks is important for a number of reasons. Firstly, *I. scapularis* is a key vector of pathogens that cause disease in humans, domestic animals, and wildlife in North America (Parola and Raoult 2001), and the species' distribution is expanding into Canada (e.g., Watson and Anderson 1976, Klich et al. 1996, Barker and Lindsay 2000, Scott et al. 2001, Morshed et al. 2005, Ogden et al. 2006c, Ogden et al. 2008c, Ogden et al. 2008d, Ogden et al. 2009, Ogden et al. 2010, Scott et al. 2010, Bouchard et al. 2011, Koffi et al. 2012, Scott et al. 2012, Mechai et al. 2013). Secondly, high county-level incidence rates of anaplasmosis, a non-specific and sometimes fatal febrile illness caused by infection with the *I. scapularis*-borne bacterium, *Anaplasma phagocytophilum* (Bakken et al. 1994, Chen et al. 1994, Hodzic et al. 1998), were reported during 2000-2007 in a cluster of several contiguous counties in the Midwest (particularly those in Minnesota and Wisconsin), whereas no such cluster was apparent in other regions of the U.S.A., including the Northeast (Dahlgren et al. 2011). Thirdly, although the number of nymphal *I. scapularis* per square km infected with *Borrelia burgdorferi*, the causative agent of Lyme disease (Burgdorfer et al. 1982, Johnson et al. 1984), did not differ significantly between the Midwest and Northeast regions of the U.S.A. (Diuk-Wasser et al. 2012), the strains of this pathogen did (e.g., Humphrey et al. 2010). Such differences are significant, as some strains of *B. burgdorferi* are more virulent to humans than others (Seinost et al. 1999). Furthermore, different strains of *B. burgdorferi* vary in their ability to survive in different reservoir hosts (Hanincova et al. 2006). Thus, an objective of this thesis was to examine the population genetics and phylogeographical structures of *I. scapularis* in order to test the hypothesis that the *I. scapularis* collected from the different regions of Canada originated from different geographical areas in the U.S.A.

While the genetic markers used in Chapters 2 and 3 were useful for resolving the relationships among the different populations of *I. scapularis*, the minimum spanning networks indicated shallow phylogeographical structures among *I. scapularis*. In Chapter 4, sequences of the D3 domain and flanking core regions (= $D3^{+}$) of the nuclear large subunit rRNA gene were compared for *I. scapularis*, resulting in the detection of a single sequence variant. In Chapter 5, the complete DNA sequence of the mt 16S rRNA gene was determined for a handful of *I. scapularis* in order to assess whether previously uncharacterized domains of this gene may be

useful for inferring the phylogeographical relationships among blacklegged ticks. The results showed that there are several regions of the mt 16S rRNA gene that might be useful for population genetics and phylogeographical studies of *I. scapularis*, one of which was investigated in the present study.

Resolving phylogeographical patterns can be challenging, as a suitable molecular marker for capturing the diversity of phylogeographical outcomes can be elusive. The ideal molecular marker is a DNA fragment that is sufficiently variable that phylogeographical patterns can be detected with relative ease, but not so highly variable as to require extraordinarily large sample sizes, make the data analysis unnecessarily complicated, or cause the phylogeographical patterns to be difficult to discern.

Mitochondrial markers are commonly used in phylogeographical studies, as animal mitochondrial (mt) DNA evolves faster than single-copy nuclear (n) DNA (Avise 2000). Many properties of animal mtDNA (e.g., maternal transmission, widespread intraspecific variation, and, in many cases, lack of intermolecular genetic recombination) make it a favourable target for phylogeographical studies (Avise 2000). To date, both mtDNA (e.g., 16S ribosomal (r) RNA, 12S rRNA, cytochrome c oxidase subunit 1 (*cox1*), cytochrome c oxidase subunit 2 (*cox2*), cytochrome c oxidase subunit 3 (*cox3*), cytochrome b (*cytb*), and the control region) and nDNA (e.g., translation elongation factor EF1-alpha (*EF1-α*), defensin, and tick receptor for the *Borrelia burgdorferi* outer surface protein A (*TROSPA*); microsatellites; and the internal transcribed spacer 2 (ITS2)) have been examined in phylogeographical studies of ticks (e.g., Qiu et al. 2002, Rosenthal and Spielman 2004, Casati et al. 2008, Kempf et al. 2009, Trout et al. 2009, Humphrey et al. 2010, Kempf et al. 2011, Krakowetz et al. 2011, Noureddine et al. 2011, Song et al. 2011, Cangi et al. 2013, Mechai et al. 2013, Dinnis et al. 2014). However, mtDNA is most commonly used (e.g., Qiu et al. 2002, Casati et al. 2008, Kempf et al. 2009, Trout et al. 2009, Humphrey et al. 2010, Krakowetz et al. 2011, Noureddine et al. 2011, Song et al. 2011, Cangi et al. 2013, Mechai et al. 2013, Dinnis et al. 2014).

In recent years, a handful of phylogeographical studies of ticks have compared genealogical structures inferred from mtDNA and nDNA, and revealed, thus far, that the phylogeographical signal of nDNA is less than that of mtDNA (Noureddine et al. 2011, Song et al. 2011, Cangi et al. 2013). Such findings are consistent with the observation that animal mtDNA evolves faster than single-copy nDNA (Avise 2000). However, in one study, a

comparison of the phylogeographical signals from microsatellite markers and those from a mitochondrial gene revealed the presence of a conspicuous population genetic structure based on microsatellites, as compared to a modest structure based on the mtDNA (Kempf et al. 2009).

Two different strategies have been used in an attempt to obtain greater phylogeographical signal from mtDNA or nDNA. The first involves examining novel DNA markers. The second is to examine concatenated datasets (e.g., Norris et al. 1996, Noureddine et al. 2011, Dinnis et al. 2014). Evidence for the efficacy of this approach is mixed. In some prior studies, the phylogenetic and phylogeographical outcomes of the concatenated datasets have been similar to the outcomes of those associated with any one of the constituent DNA sequence fragments (e.g., Norris et al. 1996, Noureddine et al. 2011). For example, a phylogenetic tree of *I. ricinus* was constructed based on a combined dataset consisting of two mitochondrial and three nuclear genes (Noureddine et al. 2011). The amalgamation of the mitochondrial and nuclear data provided limited phylogeographic information for *I. ricinus* and was comparable to the phylogenetic tree corresponding to one of the individual genes (Noureddine et al. 2011). In contrast, some studies report that concatenated datasets provide a greater phylogeographical signal than any of the individual markers. For instance, the concatenation of mtDNA from six protein-coding and ribosomal genes (i.e., adenosine triphosphate synthase subunit 6 (*atp6*), *cox1*, *cox2*, *cox3*, *cytb*, and 12S) in a more recent study of *I. ricinus* revealed a remarkable phylogeographical pattern such that for the first time, well-defined phylogeographical structure was observed in *I. ricinus* collected in Europe (Dinnis et al. 2014). This strong phylogeographical structure based on the concatenated dataset was observed despite reports of poor phylogeographical signals based on sequences of these genes, except for *apt6*, when examined individually (e.g., Casati et al. 2008, Kempf et al. 2009, Noureddine et al. 2011, Song et al. 2011, Cangi et al. 2013, Mechai et al. 2013).

The goal of this study was to obtain better phylogeographical signal for *I. scapularis* using the above two strategies in order to determine the structure of *I. scapularis* collected from different localities in the Midwest and Northeast, U.S.A. and in southern Canada. Specifically, a novel individual molecular marker (i.e., the 5' end of the mt 16S rRNA gene) was investigated, which to our knowledge has not been used in the study of any other tick species. The phylogeographical structure of *I. scapularis* was also investigated using concatenated sequence data corresponding to: 1) the 5' and 3' ends of the mt 16S rRNA gene (~700 bp), and

2) the mt 12S rRNA + $tRNA^{Val}$ genes + the 5' and 3' ends of the mt 16S rRNA gene (~1130 bp), so as to explore the strength of the phylogeographical signal offered by the concatenated markers and to test the hypothesis that the *I. scapularis* collected from the different regions of Canada originated from different geographical areas in the U.S.A.

6.3. Materials and methods

6.3.1. Samples

A total of 582 adult and nymphal *I. scapularis* were collected from hosts or the environment between 2000 and 2011. Details regarding the collection of these ticks have already been summarized in Chapter 2. However, in the present study, one fewer tick (i.e., a female) from Saskatchewan (SK) and one additional tick (i.e., a male) from Long Point Provincial Park, Ontario (ON) were examined. Two adult *I. scapularis* (i.e., one from Point Pelee National Park, ON and another from Lunenburg, Nova Scotia (NS)) collected for use in a prior study (Krakowetz et al. 2011) were also included.

6.3.2. Molecular analyses

Total genomic (g) DNA was extracted and purified from a total of 584 individual *I. scapularis* using the procedure described in Chapter 2 for 582 ticks or the methodology described in the study by Krakowetz et al. (2011) for two ticks. An ~325 bp region starting at the 3' end the $tRNA^{Val}$ gene and ending within Domain II of the mt 16S rRNA gene was amplified by PCR from the total gDNA of each tick using the primers Iscap-tRNA-Val (5'-ACAATGAAA ATTTTGTTGTTTAACC-3') and Iscap-16S-New-1 (5'-ACCAGATATCATTAAATATG-3'), which were designed based on a comparison of DNA sequences from the mitochondrial genomes of *I. ricinus* (GenBank accession no. JN248424) and *Ixodes persulcatus* (GenBank accession no. AB073725). These two tick species, as well as *I. scapularis*, are members of the subgenus *Ixodes* (Xu et al. 2003). Amplicons from each tick individual (n = 453) were obtained via PCRs that were conducted in 25 µl volumes consisting of 1X *Taq* Buffer with KCl (Fermentas, Fisher Scientific, Ottawa, ON, Canada), 200 µM of each dNTP (Fermentas), 1.75 mM $MgCl_2$ (Fermentas), 1 uM of each primer, 0.5 U of *Taq* DNA Polymerase (Fermentas), and 1 or 1.5 µl of gDNA template. A negative control containing UltraPure™ DNase/RNase-Free Distilled Water

(Invitrogen™, Life Technologies Inc., Burlington, ON, Canada) rather than total gDNA was included in each set of PCRs. The thermocycler conditions used were: 96ºC for 5 min, then 30 cycles of 94ºC for 30 s, 54ºC for 30 s, and 72ºC for 30 s, followed by a final 5 min extension at 72ºC. Amplicons were subjected to electrophoresis at 120 V for 30 or 45 min on 1.5% (w/v) agarose-TBE gels (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3; OmniPur®, EMD Millipore Ltd., Etobicoke, ON, Canada), which were stained with SYBR® Safe DNA Gel Stain (Life Technologies Inc., Burlington, ON, Canada).

Subsequently, single-strand conformation polymorphism (SSCP) analyses were employed to screen amplicons for sequence variability. The methodology followed that of Krakowetz et al. (2011), except that 1-5 µl of the amplicons from each PCR was mixed with 0-4 µl of DNase-free water and 5 µl of loading buffer (Gel Tracking Dye™; Promega, Madison, U.S.A.) and the resultant mixture was electrophoresed for 15 h. The SSCP profiles (i.e., banding patterns) of the different tick individuals were reproducible on the same or different gel(s) or day(s). However, due to the large number of SSCP profiles representing the 453 *I. scapularis* individuals relative to the small number of wells (i.e., 25 lanes) available on a single SSCP gel, line-up gels were used to confirm the SSCP profiles of many samples. The amplicons of at least two ticks representing the same banding pattern were purified (see Chapter 2 for the methodology used) and subjected to automated DNA sequencing in order to confirm that their sequences were identical. Amplicons representing different banding patterns were also purified and sequenced. Sequencing was carried out using the primers Iscap-tRNA-Val (208 samples) or Iscap-16S-New-1 (255 samples) in separate reactions.

6.3.3. Data analyses

The DNA sequences of Domains I and II of the mt 16S rRNA gene were manually aligned, but modified according to the predicted secondary structure of the rRNA that was constructed for *I. scapularis* (see Chapter 5) based on secondary structure models for other arthropods (Gutell and Fox 1988, Gutell et al. 1993, Gutell 1996). The sequence data of the 5' end (i.e., Domains I and II) of the mt 16S rRNA gene (this study) were then concatenated with the sequence data of the 3' end (i.e., Domains IV and V) of the mt 16S rRNA gene (Chapter 2 or Krakowetz et al. 2011) for each tick (n = 576). Subsequently, the sequence data of the 3' end of

the mt 12S rRNA gene and the complete transfer (t) RNA^{Val} gene (Chapter 3) were concatenated with the combined data of the 5' and 3' ends of the mt 16S rRNA gene for 225 ticks.

The sequence data of the 5' end of the mt 16S rRNA gene was used to estimate haplotype richness in the "eastern" (i.e., Point Pelee National Park (PPNP) and Long Point Provincial Park (LPPP) in ON; and Trustom Pond, South Kingstown (TPSK) and Hazard Island, South Kingstown (HISK) in Rhode Island (RI)) and "western" (i.e., Pembina Valley Provincial Park (PVPP) and Stanley Trail (ST) in Manitoba (MB), and Itasca State Park (ISP), Camp Ripley (CR), and St. Croix State Park (CSP) in Minnesota (MN)) geographical regions. Interpolation (i.e., rarefaction) and extrapolation curves, as well as their 95% confidence intervals, were generated for each region using the computer program EstimateS (Colwell 2013). For each geographical area, sample-based abundance data (i.e., haplotype frequency data) was input as a single set of replicated sampling units. To estimate the total haplotype richness in each of the "eastern" and "western" geographical regions, rarefaction and extrapolation curves, and their respective 95% confidence intervals, were constructed using EstimateS with sample-based abundance data and the classic version of the non-parametric estimator Chao 2 with 1000 randomizations. The above analyses were also conducted on the sequence data for the nine established populations. The classic version of Chao 2 with 1000 randomizations was used for this dataset. For comparative purposes, a scatter plot of the number of haplotypes observed at each established population as a function of the total number of ticks collected from that population was also created.

A minimum spanning network (i.e., haplotype network) was constructed using the computer program TCS (Clement et al. 2000) to depict the relationships of the different haplotypes of Domains I and II of the mt 16S rRNA gene. Haplotype networks were also constructed for the concatenated datasets.

6.4. Results

6.4.1. The mt 16S rRNA gene (Domains I and II)

6.4.1.1. Molecular and sequence analyses

A single band of the expected size (i.e., ~325 bp) was detected upon electrophoresis for each tick (n = 453) when amplicons of Domains I and II of the mt 16S rRNA gene were run on agarose gels (Fig. 6.1). Bands were not detected for the negative (i.e., no gDNA template) controls (Fig. 6.1). There were dozens of different profiles (i.e., banding patterns) among the amplicons of ~325 bp when subjected to SSCP analyses (Fig. 6.2). Line-up gels were used to facilitate the discrimination of SSCP profiles that appeared to be the same or similar on an SSCP gel and to distinguish SSCP profiles corresponding to samples run on different gels and/or different days (Fig. 6.3). Amplicons with the same SSCP profile were identical in DNA sequence, whereas those with differing profiles varied from one another by at least 1 bp. Sequences corresponding to representative SSCP profiles were aligned with those obtained using the primers Tick-12S-1 and/or Iscap-16S-New-1 in the study described in Chapter 3 over 286 bp. The aligned sequences represented a total of 130 sequence variants (i.e., haplotypes) and differed from one another by 1-17 bp (Table 6.1). There were 74 variable positions in the alignment of the 130 haplotypes (Table 6.1). These mutational differences were comprised of 13 purine and 10 pyrimidine transitions, 21 transversions, 14 indels, and 16 multiple mutational changes.

6.4.1.2. Genetic variation

Table 6.2 shows the number of *I. scapularis* of the 130 different mt 16S rRNA gene haplotypes that were collected from several provinces in Canada and two states (MN and RI) in the U.S.A. The most common haplotype was Hap_2, which represented 23.1% of the 577 ticks. The next seven most common haplotypes (i.e., Hap_1, Hap_7, Hap_26, Hap_34, Hap_45, Hap_52, and Hap_72) were each represented by 2.1-7.1% of the ticks examined and, together, comprised 24.1% of the *I. scapularis*. A total of 60 haplotypes were each represented by a single tick (i.e., singletons), and 26 haplotypes were each represented by two ticks (i.e., doubletons). These rare haplotypes, which comprised 19.4% of the 577 blacklegged ticks studied, represented 66.2% of the 130 total haplotypes. Twelve haplotypes (i.e., Hap_1, Hap_2, Hap_20, Hap_22,

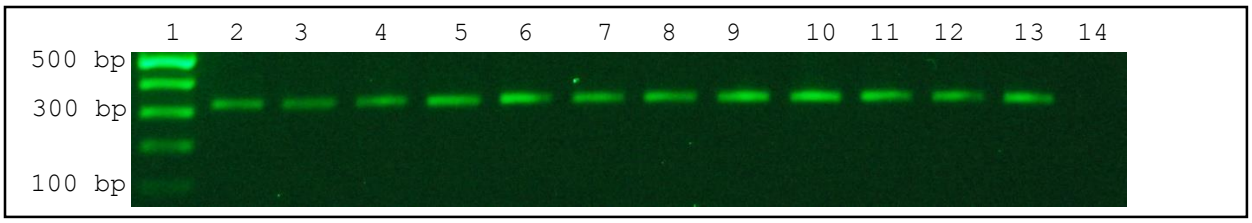


Figure 6.1. Agarose gel showing the amplicons (~325 bp) produced by PCR from the total genomic (g) DNA of individual *I. scapularis* collected from Minnesota, U.S.A. (lane 2) and Manitoba, Canada (lanes 3-13) using the primers Iscap-tRNA-Val and Iscap-16S-New1, which were designed for use in the present study. Included in this set of PCRs was a negative (i.e., no gDNA) control (lane 14). The GeneRuler™ 100 bp Plus DNA Ladder 100 to 3000 bp (Thermo Fisher Scientific, Ottawa, ON, Canada) was used as a size standard (lane 1).

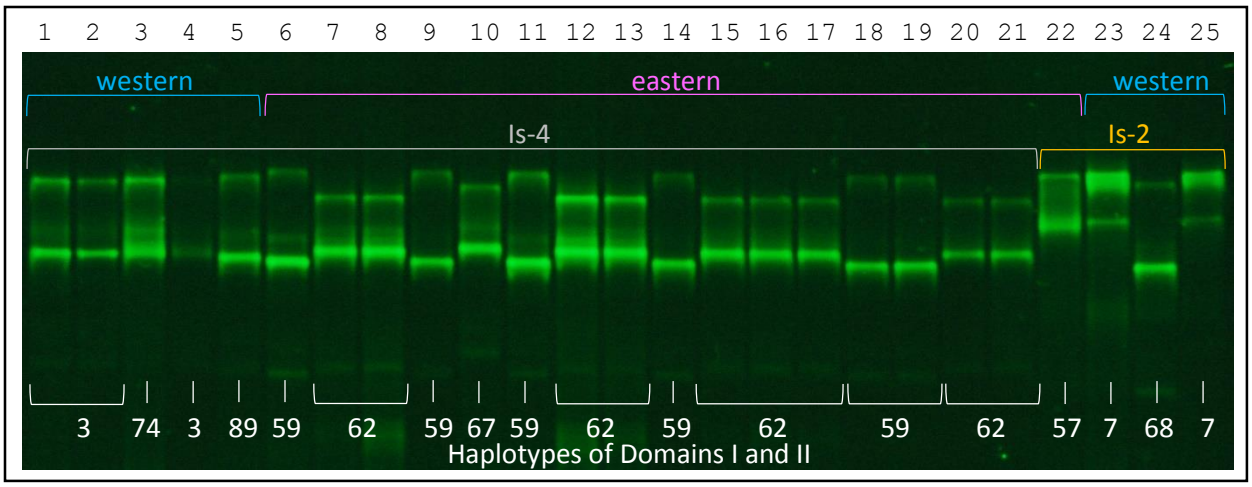


Figure 6.2. SSCP gel showing the banding patterns of some amplicons of Domains I and II of the mt 16S rRNA gene produced by PCR from the total genomic (g) DNA of individual *I. scapularis* collected from Manitoba (lanes 1-5 and 25), Quebec (lanes 6-7 and 9), Prince Edward Island (lanes 8 and 10), Ontario (lanes 11 and 22), and Newfoundland (lane 12) in Canada, and Rhode Island (lanes 13-21) and Minnesota (lanes 23 and 24) in the U.S.A. using the primers Iscap-tRNA-Val and Iscap-16S-New1, which were designed for use in the present study. The banding patterns on this gel represent nine haplotypes (white text) of Domains I and II of the mt 16S rRNA gene. *I. scapularis* individuals previously characterized as haplotype Is-4 based on their sequences of Domains IV and V of the mt 16S rRNA gene are represented in lanes 1-21 (grey text), while those previously characterized as haplotype Is-2 are represented in lanes 22-25 (orange-yellow text). Amplicons of ticks collected from the "western" and "eastern" geographical regions, as defined in Chapter 2, are shown in lanes 1-5 and 23-25 (blue text), and 6-22 (pink text), respectively.

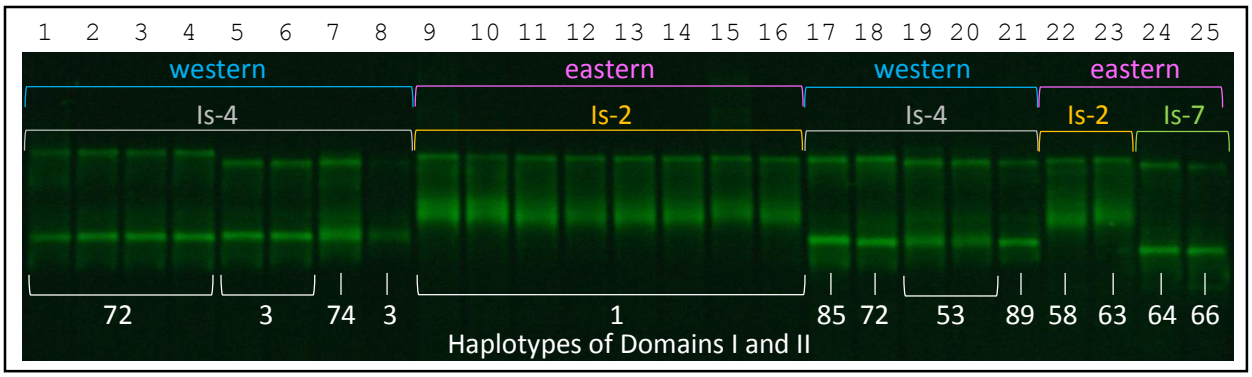


Figure 6.3. SSCP gel showing the similar banding patterns of some amplicons of Domains I and II of the mt 16S rRNA gene produced by PCR from the total genomic (g) DNA of individual *I. scapularis* collected from Manitoba (lanes 1-8 and 19-21), Quebec (lane 9), and New Brunswick (lane 10) in Canada, and Minnesota (lanes 17 and 18) and Rhode Island (lanes 11-16 and 22-25) in the U.S.A. using the primers Iscap-tRNA-Val and Iscap-16S-New1, which were designed for use in the present study. The banding patterns on this gel represent 11 haplotypes (white text) of Domains I and II of the mt 16S rRNA gene. *I. scapularis* individuals previously characterized as haplotype Is-4 based on their sequences of Domains IV and V of the mt 16S rRNA gene are represented in lanes 1-8 and 17-21 (grey text), while those previously characterized as haplotype Is-2 are represented in lanes 9-16, 22, and 23 (orange-yellow text). Lanes 24 and 25 contain two *I. scapularis* individuals formerly characterized as haplotype Is-7 of Domains IV and V of the mt 16S rRNA gene (green text). Amplicons of ticks collected from the "western" and "eastern" geographical regions, as defined in Chapter 2, are shown in lanes 1-8 and 17-21 (blue text), and 9-16 and 22-25 (pink text), respectively.

Table 6.1. Variable positions in the 286 bp alignment of the DNA sequence variants (i.e., haplotypes (HT)). A dot (.) at an alignment position indicates the same nucleotide as in the sequence of Hap_2, while a dash (-) represents a deletion. The 5' end of the mt 16S rRNA gene was the molecular marker used to characterize 577 *I. scapularis* individuals.

| HT | Alignment position: | | |

Table 6.1. Continued.

| HT | Alignment position: | | |

Table 6.1. Continued.

| HT | Alignment position: | | |

Table 6.1. Continued.

| HT | Alignment position: | | |

Table 6.2. The number of *I. scapularis* of the different mt 16S rRNA gene haplotypes collected from nine provinces* in Canada and two states (MN and RI) in the U.S.A.

Haplotype	*n*	No. individuals from:										
		AB	SK	MB	MN	ON	QC	RI	NB	PE	NS	NL
Hap_1	25	0	1	0	2	13	2	6	1	0	0	0
Hap_2	133	0	0	19	45	48	2	16	0	0	3	0
Hap_3	9	0	0	8	1	0	0	0	0	0	0	0
Hap_4	2	0	0	2	0	0	0	0	0	0	0	0
Hap_5	7	0	1	2	4	0	0	0	0	0	0	0
Hap_6	1	0	0	1	0	0	0	0	0	0	0	0
Hap_7	18	0	0	11	7	0	0	0	0	0	0	0
Hap_8	1	0	0	0	1	0	0	0	0	0	0	0
Hap_9	1	0	0	1	0	0	0	0	0	0	0	0
Hap_10	1	0	0	0	1	0	0	0	0	0	0	0
Hap_11	1	0	0	0	1	0	0	0	0	0	0	0
Hap_12	1	0	0	1	0	0	0	0	0	0	0	0
Hap_13	3	0	0	0	3	0	0	0	0	0	0	0
Hap_14	4	0	1	0	0	0	0	2	0	0	1	0
Hap_15	1	0	0	1	0	0	0	0	0	0	0	0
Hap_16	2	0	0	1	1	0	0	0	0	0	0	0
Hap_17	4	0	0	1	3	0	0	0	0	0	0	0
Hap_18	2	0	0	2	0	0	0	0	0	0	0	0
Hap_19	2	0	0	1	1	0	0	0	0	0	0	0
Hap_20	6	0	0	1	1	2	1	0	0	0	1	0
Hap_21	3	0	0	0	3	0	0	0	0	0	0	0
Hap_22	9	0	0	2	2	2	1	2	0	0	0	0
Hap_23	2	0	0	0	0	2	0	0	0	0	0	0
Hap_24	1	0	0	0	1	0	0	0	0	0	0	0
Hap_25	4	0	0	1	3	0	0	0	0	0	0	0
Hap_26	12	0	0	0	0	12	0	0	0	0	0	0
Hap_27	1	0	0	1	0	0	0	0	0	0	0	0
Hap_28	1	0	0	1	0	0	0	0	0	0	0	0
Hap_29	5	0	0	0	5	0	0	0	0	0	0	0
Hap_30	1	0	0	0	1	0	0	0	0	0	0	0
Hap_31	1	0	0	1	0	0	0	0	0	0	0	0
Hap_32	1	0	0	0	1	0	0	0	0	0	0	0
Hap_33	6	0	0	0	0	0	1	5	0	0	0	0
Hap_34	17	0	0	0	1	1	4	8	0	1	2	0
Hap_35	6	0	0	2	1	0	1	2	0	0	0	0
Hap_36	1	0	0	0	0	0	0	1	0	0	0	0
Hap_37	4	0	0	0	0	0	0	4	0	0	0	0
Hap_38	4	0	0	0	4	0	0	0	0	0	0	0
Hap_39	9	0	0	2	5	1	0	1	0	0	0	0
Hap_40	1	0	0	0	0	1	0	0	0	0	0	0
Hap_41	1	0	0	0	0	0	1	0	0	0	0	0
Hap_42	1	0	0	0	0	0	0	1	0	0	0	0
Hap_43	3	0	0	0	3	0	0	0	0	0	0	0
Hap_44	1	0	0	0	0	0	0	0	0	0	1	0
Hap_45	41	1	0	1	2	35	0	1	1	0	0	0
Hap_46	1	0	0	0	0	0	0	1	0	0	0	0
Hap_47	1	0	0	0	0	0	1	0	0	0	0	0
Hap_48	1	0	0	0	0	1	0	0	0	0	0	0
Hap_49	1	0	0	0	0	0	0	1	0	0	0	0
Hap_50	4	0	0	0	4	0	0	0	0	0	0	0

Table 6.2. Continued.

Haplotype	*n*	No. individuals from:										
		AB	SK	MB	MN	ON	QC	RI	NB	PE	NS	NL
Hap_51	5	0	0	0	5	0	0	0	0	0	0	0
Hap_52	14	0	0	0	0	1	0	11	0	0	1	1
Hap_53	2	0	0	2	0	0	0	0	0	0	0	0
Hap_54	1	0	0	0	0	0	0	1	0	0	0	0
Hap_55	1	0	0	0	0	0	0	1	0	0	0	0
Hap_56	1	0	0	0	0	0	0	1	0	0	0	0
Hap_57	2	0	0	0	0	2	0	0	0	0	0	0
Hap_58	1	0	0	0	0	0	0	1	0	0	0	0
Hap_59	10	0	1	0	0	1	2	6	0	0	0	0
Hap_60	1	0	0	0	0	0	0	1	0	0	0	0
Hap_61	2	0	0	0	0	0	0	1	0	0	1	0
Hap_62	9	0	0	0	0	0	1	6	0	1	0	1
Hap_63	4	0	0	0	3	0	0	1	0	0	0	0
Hap_64	2	0	0	0	0	0	0	2	0	0	0	0
Hap_65	1	0	0	0	0	1	0	0	0	0	0	0
Hap_66	10	0	0	0	0	1	0	9	0	0	0	0
Hap_67	1	0	0	0	0	0	0	0	0	1	0	0
Hap_68	3	0	0	2	1	0	0	0	0	0	0	0
Hap_69	1	0	0	0	1	0	0	0	0	0	0	0
Hap_70	1	0	0	0	0	0	0	1	0	0	0	0
Hap_71	1	0	0	0	1	0	0	0	0	0	0	0
Hap_72	12	0	0	6	6	0	0	0	0	0	0	0
Hap_73	1	0	0	0	1	0	0	0	0	0	0	0
Hap_74	2	0	0	1	1	0	0	0	0	0	0	0
Hap_75	7	0	0	0	0	0	4	1	1	0	1	0
Hap_76	5	0	0	2	3	0	0	0	0	0	0	0
Hap_77	1	0	0	0	0	0	0	0	1	0	0	0
Hap_78	1	0	0	0	0	0	1	0	0	0	0	0
Hap_79	2	1	0	0	1	0	0	0	0	0	0	0
Hap_80	1	0	0	0	1	0	0	0	0	0	0	0
Hap_81	1	0	0	0	1	0	0	0	0	0	0	0
Hap_82	1	0	0	0	1	0	0	0	0	0	0	0
Hap_83	10	0	0	0	10	0	0	0	0	0	0	0
Hap_84	2	0	0	0	2	0	0	0	0	0	0	0
Hap_85	3	0	0	2	1	0	0	0	0	0	0	0
Hap_86	2	0	0	2	0	0	0	0	0	0	0	0
Hap_87	2	0	1	0	1	0	0	0	0	0	0	0
Hap_88	1	0	0	0	1	0	0	0	0	0	0	0
Hap_89	1	0	0	1	0	0	0	0	0	0	0	0
Hap_90	1	0	0	0	1	0	0	0	0	0	0	0
Hap_91	1	0	0	0	1	0	0	0	0	0	0	0
Hap_92	3	0	0	0	3	0	0	0	0	0	0	0
Hap_93	2	0	0	0	2	0	0	0	0	0	0	0
Hap_94	1	0	0	0	0	1	0	0	0	0	0	0
Hap_95	3	0	0	0	0	3	0	0	0	0	0	0
Hap_96	4	0	0	0	0	4	0	0	0	0	0	0
Hap_97	5	0	0	0	0	5	0	0	0	0	0	0
Hap_98	3	0	0	0	0	3	0	0	0	0	0	0
Hap_99	1	0	0	0	0	1	0	0	0	0	0	0
Hap_100	1	0	0	0	0	1	0	0	0	0	0	0

Table 6.2. Continued.

Haplotype	*n*	No. individuals from:										
		AB	SK	MB	MN	ON	QC	RI	NB	PE	NS	NL
Hap_101	2	0	0	0	0	2	0	0	0	0	0	0
Hap_102	2	0	0	0	0	2	0	0	0	0	0	0
Hap_103	1	0	0	0	0	1	0	0	0	0	0	0
Hap_104	2	0	0	0	0	2	0	0	0	0	0	0
Hap_105	1	0	0	0	0	1	0	0	0	0	0	0
Hap_106	1	0	1	0	0	0	0	0	0	0	0	0
Hap_107	1	0	0	0	1	0	0	0	0	0	0	0
Hap_108	1	0	0	0	0	0	0	1	0	0	0	0
Hap_109	2	0	0	0	2	0	0	0	0	0	0	0
Hap_110	4	0	0	2	0	2	0	0	0	0	0	0
Hap_111	1	0	0	0	1	0	0	0	0	0	0	0
Hap_112	4	0	0	3	1	0	0	0	0	0	0	0
Hap_113	1	0	0	0	0	0	0	1	0	0	0	0
Hap_114	2	0	0	0	0	0	0	1	1	0	0	0
Hap_115	2	0	0	1	0	1	0	0	0	0	0	0
Hap_116	1	0	0	1	0	0	0	0	0	0	0	0
Hap_117	6	0	0	5	1	0	0	0	0	0	0	0
Hap_118	1	0	0	0	1	0	0	0	0	0	0	0
Hap_119	2	0	0	1	1	0	0	0	0	0	0	0
Hap_120	2	0	0	0	0	0	0	0	1	0	1	0
Hap_121	2	0	0	0	0	2	0	0	0	0	0	0
Hap_122	2	0	0	0	0	2	0	0	0	0	0	0
Hap_123	1	0	0	0	0	1	0	0	0	0	0	0
Hap_124	1	0	0	0	0	0	0	1	0	0	0	0
Hap_125	1	0	0	0	1	0	0	0	0	0	0	0
Hap_126	2	0	0	0	2	0	0	0	0	0	0	0
Hap_127	1	0	0	0	1	0	0	0	0	0	0	0
Hap_128	1	0	0	1	0	0	0	0	0	0	0	0
Hap_129	5	0	0	0	0	5	0	0	0	0	0	0
Hap_130	1	0	0	0	0	1	0	0	0	0	0	0
Total	577	2	6	95	167	164	22	98	6	3	12	2

* AB = Alberta, SK = Saskatchewan, MB = Manitoba, ON = Ontario, QC = Quebec, NB = New Brunswick, PE = Prince Edward Island, NS = Nova Scotia, and NL = Newfoundland.

Hap_34, Hap_35, Hap_39, Hap_45, Hap_52, Hap_59, Hap_62, and Hap_75) were present in 4-6 provinces/states. In contrast, 92 haplotypes were detected in a single geographical area only.

The interpolation (i.e., rarefaction) and extrapolation curves for the "western" and "eastern" populations did not reach their respective asymptotes, but showed that the estimated number of haplotypes in the "western" populations was greater than that in the "eastern" populations; however, this difference was not statistically significant, as there was overlap in the 95% confidence intervals of the two curves (Fig. 6.4). It was not possible to compare the haplotype richness of the "western" and "eastern" populations, as the Chao 2 curve for the "eastern" populations did not reach an asymptote. However, the Chao 2 estimator revealed that the haplotype richness of the "western" populations was 126 haplotypes. The rarefaction and extrapolation curves for the combined population data did not reach an asymptote, and the Chao 2 estimate of haplotype richness for the nine established populations was 250 haplotypes (Fig. 6.5). The scatter plot in Fig. 6.5 showed that more haplotypes were detected in each of the populations in the "western" and "eastern" geographical areas than would be expected for a given sample size (i.e., most points laid on or above the curve), except for in the populations PPNP and LPPP, ON, where fewer haplotypes were detected than would be expected for the sample sizes of 47 and 105 ticks, respectively (i.e., the points corresponding to PPNP and LPPP laid beyond the 95% confidence interval).

6.4.1.3. Phylogeographical analyses

Fig. 6.6 depicts the relationships among the 130 haplotypes (see Tables 6.1 and 6.2). A "star" phylogeny was represented in the minimum spanning network (e.g., Avise 2000, Qiu et al. 2002, Humphrey et al. 2010). "Starburst" and "chain" configurations were also present. The most common and widespread haplotype (i.e., Hap_2) represented the central haplotype of the "star" phylogeny from which 22 (i.e., 16.9%) of the 130 haplotypes directly differed from by 1 bp. Similarly, "starburst" patterns consisted of a "central" haplotype (e.g., Hap_1, Hap_52, and Hap_72) from which several haplotypes were connected to and differed from by one or more base pairs. The "chain" pattern (i.e., ≥3 haplotypes linearly linked; no branching) consisted of the following known haplotypes: Hap_81 (which was linked to Hap_1 through Hap_68), Hap_12, Hap_34, and Hap_49. Most (i.e., 65) haplotypes in the network were represented by ticks that were collected from the "western" geographical region only; however, a total of 52 haplotypes

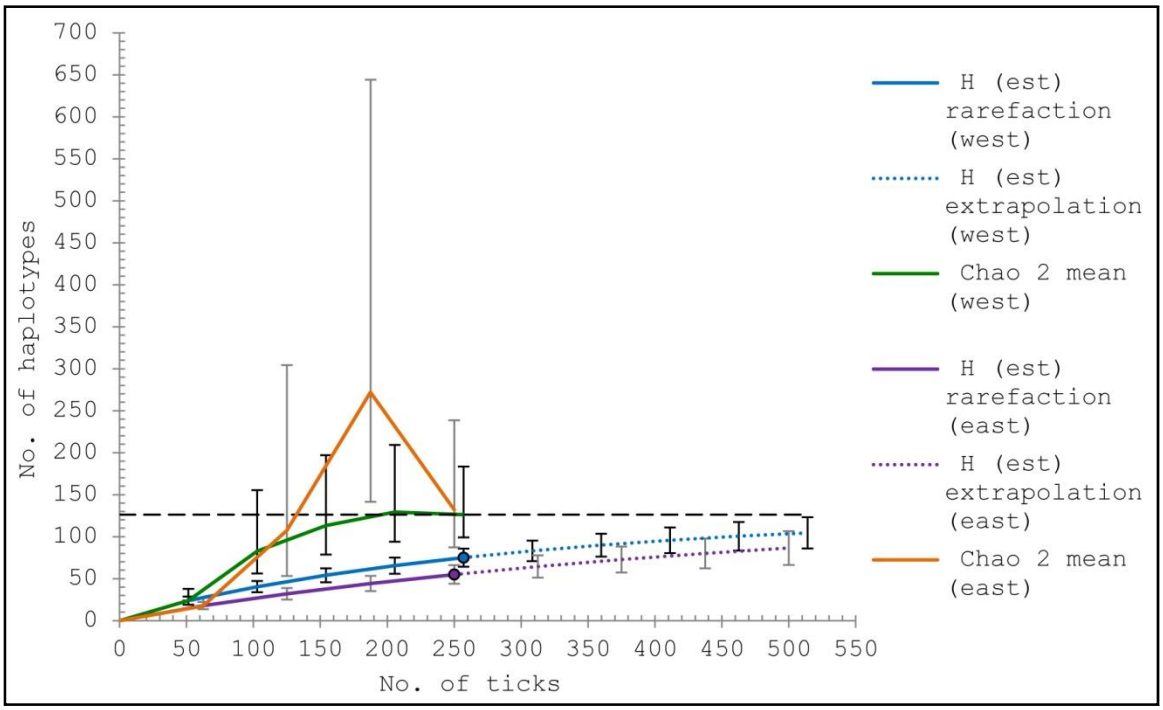


Figure 6.4. Rarefaction, extrapolation, and estimated total haplotype richness (Chao 2) curves with 95% confidence intervals for the five established populations of *I. scapularis* in the "western" (Pembina Valley Provincial Park, Stanley Trail, Itasca State Park, Camp Ripley, and St. Croix State Park) and for the four in the "eastern" (Point Pelee National Park, Long Point Provincial Park, Trustom Pond, South Kingstown, and Hazard Island, South Kingstown) areas of the U.S.A. and Canada. The asymptote of the estimated total haplotype richness curve for the "western" populations is denoted by the black dashed line. The estimated total haplotype richness curve for the "eastern" populations does not appear to reach an asymptote.

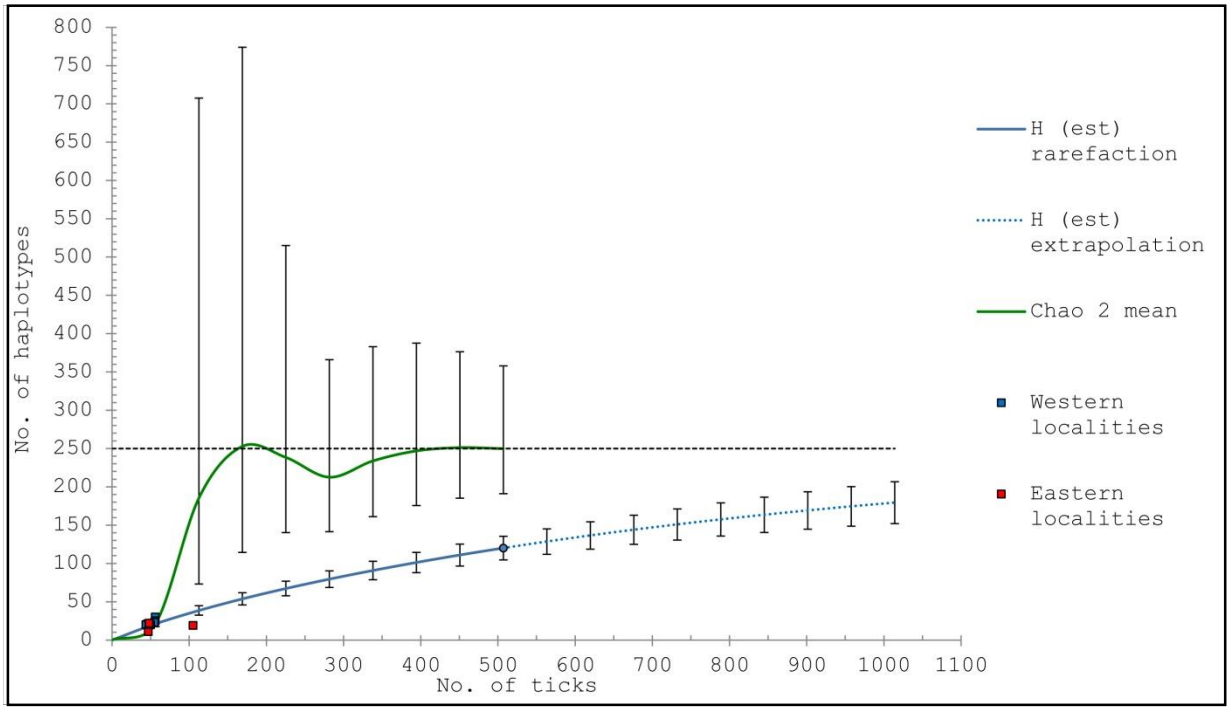


Figure 6.5. Rarefaction, extrapolation, and estimated total haplotype richness (Chao 2) curves with 95% confidence intervals for the nine established populations of *I. scapularis* in the U.S.A. and Canada, and scatter plot of the number of haplotypes found at each population against the total number of ticks collected from each. The asymptote of the estimated total haplotype richness curve is denoted by the black dashed line.

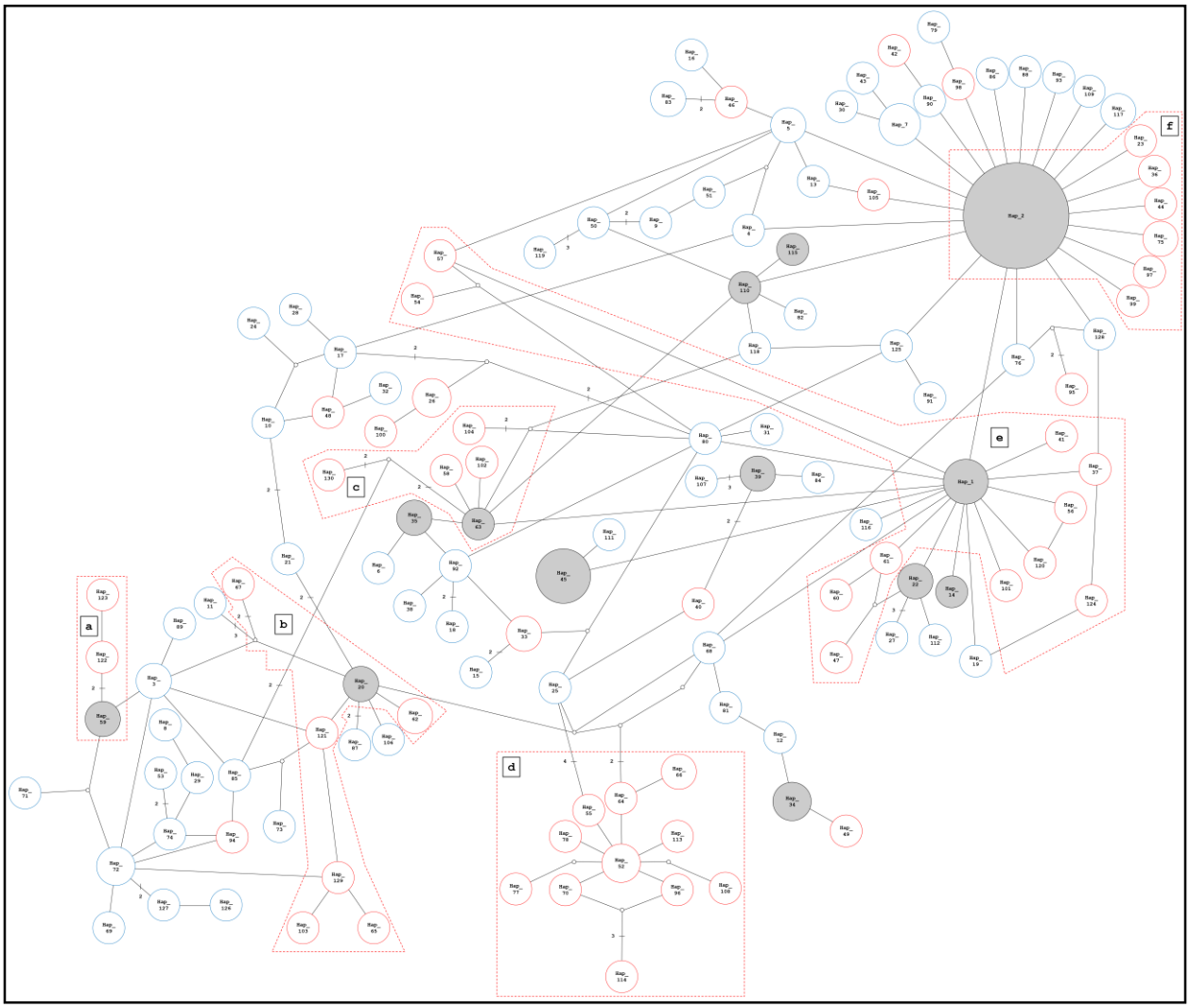


Figure 6.6. A minimum spanning network depicting the relationships among haplotypes of Domains I and II of the mt 16S rRNA gene of *I. scapularis* detected in the present study. Numerical designations, which correspond to those in Tables 6.1 and 6.2, have been assigned to the 130 haplotypes. The number of nucleotide differences between pairs of DNA sequences (circles) is one unless otherwise indicated. The size of each circle is proportional to the number of ticks of that haplotype. Putative haplotypes are represented by small open circles. Haplotypes are denoted by blue, grey, or red circles depending on whether each was detected in the "western," "western" and "eastern," or "eastern" geographical areas, respectively. Several relatively distinct phylogroups (a-f), each of which is comprised of haplotypes corresponding to the "eastern" geographical region, are contained within the red polygons.

were detected in the "eastern" geographical region only. Thirteen haplotypes (i.e., Hap_1, Hap_2, Hap_14, Hap_20, Hap_22, Hap_34, Hap_35, Hap_39, Hap_45, Hap_59, Hap_63, Hap_110, and Hap_115) were detected in both the "western" and "eastern" geographical regions. Several relatively distinct phylogroups (i.e., phylogroups a-f) were identified that corresponded to haplotypes of the "eastern" geographical region. One phylogroup (i.e., phylogroup d, which was comprised of Hap_52 and its associated haplotypes) was particularly distinct from the other haplotypes in the network.

The Venn diagram in Fig. 6.7 reveals the number of haplotypes that were detected in each of the four geographical areas: "western" Canada, "eastern" Canada, "western" U.S.A., and "eastern" U.S.A. A total of 59 haplotypes were detected from ticks collected in the Upper Midwest (i.e., Minnesota) of the U.S.A., whereas only 43 haplotypes were identified from ticks collected in the Prairie Provinces (i.e., Alberta (AB), SK, and Manitoba (MB)) of Canada. Twenty-four haplotypes were detected in both the Prairie Provinces and Minnesota. The number of haplotypes detected in Central (i.e., ON and Quebec (QC)) and Atlantic (i.e., Newfoundland, Prince Edward Island, New Brunswick, and NS) Canada was 49, while the number present in the Northeast (i.e., Rhode Island) of the U.S.A. was the fewest of the four regions at 32. A total of 16 haplotypes that were found in Rhode Island were also found in "eastern" (i.e., Central and Atlantic) Canada. In the U.S.A., the number of haplotypes in common between the Upper Midwest and Northeast regions was eight. In Canada, the number of haplotypes in common between the Prairie Provinces and "eastern" Canada was 11. Six haplotypes (i.e., Hap_1, Hap_2, Hap_22, Hap_35, Hap_39, and Hap_45) were present in all four geographical areas.

6.4.2. Concatenated datasets

6.4.2.1. Genetic variation

Analysis of the concatenated sequence data (695 bp total) of Domains I and II (this chapter) and IV and V (Chapter 2 and Krakowetz et al. 2011) of the mt 16S rRNA gene for 576 adult and nymphal *I. scapularis* showed a total of 179 haplotypes among the ticks (Table 6.3). The most common haplotype was Is-1 + Hap_2, which represented 16.1% of the ticks collected, whereas the next six most common haplotypes (i.e., Is-15 + Hap_45, Is-1 + Hap_34, Is-7 + Hap_52, Is-63 + Hap_45, Is-1 + Hap_26, and Is-13 + Hap_2) comprised only 2.1-3.8% of them.

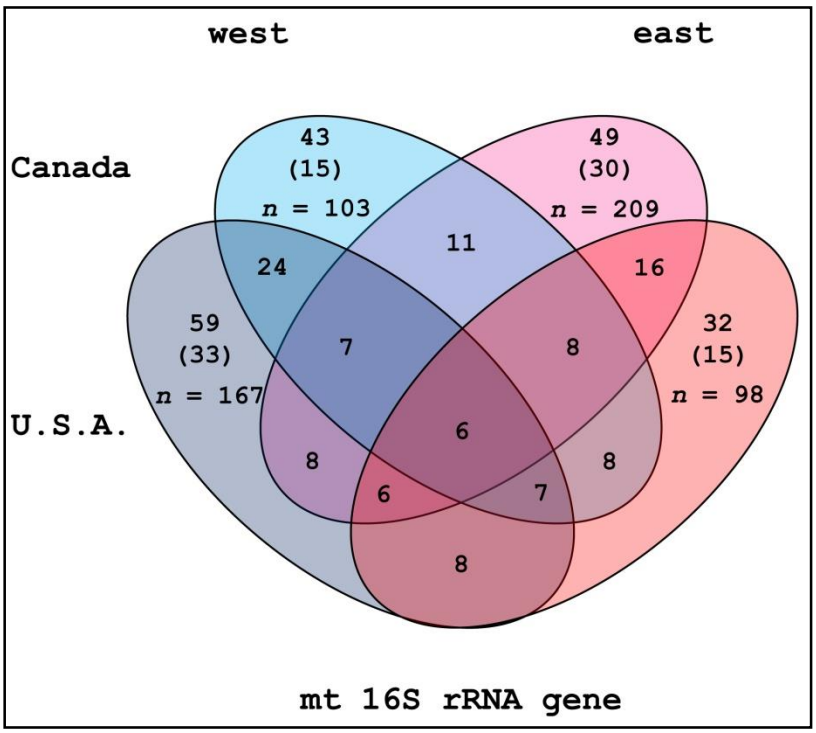


Figure 6.7. Venn diagram representing the number of haplotypes of Domains I and II of the mt 16S rRNA gene found in and shared among key geographical areas. Ovals correspond to: "western" Canada (light blue), "eastern" Canada (pink), "western" U.S.A. (dark blue), and "eastern" U.S.A. (red). The number of haplotypes that were found within a particular geographical region only (in parentheses), as well as the sample size (*n*) corresponding to each region are also reported. Haplotypes of the *I. scapularis* collected from the nine established populations (*n* = 507) and those of the adventitious ticks (*n* = 70) have been included.

Table 6.3. The number of *I. scapularis* individuals of each haplotype based on the concatenated data for the 3' (Is series) and 5' (Hap series) ends of the mt 16S rRNA gene. The haplotype based on the 3' end of the mt 16S rRNA gene of each tick was determined in Chapter 2 or by Krakowetz et al. (2011).

16S haplotype:		*n*	No. individuals from:*										
3'	5'		AB	SK	MB	MN	ON	QC	RI	NB	PE	NS	NL
Is-1	Hap_1	7	0	0	0	0	7	0	0	0	0	0	0
Is-1	Hap_2	93	0	0	13	28	34	0	15	0	0	3	0
Is-1	Hap_3	2	0	0	2	0	0	0	0	0	0	0	0
Is-1	Hap_4	2	0	0	2	0	0	0	0	0	0	0	0
Is-1	Hap_5	6	0	1	2	3	0	0	0	0	0	0	0
Is-1	Hap_7	10	0	0	6	4	0	0	0	0	0	0	0
Is-1	Hap_8	1	0	0	0	1	0	0	0	0	0	0	0
Is-1	Hap_9	1	0	0	1	0	0	0	0	0	0	0	0
Is-1	Hap_10	1	0	0	0	1	0	0	0	0	0	0	0
Is-1	Hap_11	1	0	0	0	1	0	0	0	0	0	0	0
Is-1	Hap_12	1	0	0	1	0	0	0	0	0	0	0	0
Is-1	Hap_13	3	0	0	0	3	0	0	0	0	0	0	0
Is-1	Hap_14	1	0	1	0	0	0	0	0	0	0	0	0
Is-1	Hap_15	1	0	0	1	0	0	0	0	0	0	0	0
Is-1	Hap_16	2	0	0	1	1	0	0	0	0	0	0	0
Is-1	Hap_17	4	0	0	1	3	0	0	0	0	0	0	0
Is-1	Hap_18	2	0	0	2	0	0	0	0	0	0	0	0
Is-1	Hap_19	2	0	0	1	1	0	0	0	0	0	0	0
Is-1	Hap_20	3	0	0	0	1	2	0	0	0	0	0	0
Is-1	Hap_21	3	0	0	0	3	0	0	0	0	0	0	0
Is-1	Hap_22	8	0	0	1	2	2	1	2	0	0	0	0
Is-1	Hap_23	2	0	0	0	0	2	0	0	0	0	0	0
Is-1	Hap_24	1	0	0	0	1	0	0	0	0	0	0	0
Is-1	Hap_25	4	0	0	1	3	0	0	0	0	0	0	0
Is-1	Hap_26	12	0	0	0	0	12	0	0	0	0	0	0
Is-1	Hap_27	1	0	0	1	0	0	0	0	0	0	0	0
Is-1	Hap_28	1	0	0	1	0	0	0	0	0	0	0	0
Is-1	Hap_29	5	0	0	0	5	0	0	0	0	0	0	0
Is-1	Hap_30	1	0	0	0	1	0	0	0	0	0	0	0
Is-1	Hap_31	1	0	0	1	0	0	0	0	0	0	0	0
Is-1	Hap_32	1	0	0	0	1	0	0	0	0	0	0	0
Is-1	Hap_33	6	0	0	0	0	0	1	5	0	0	0	0
Is-1	Hap_34	16	0	0	0	0	1	4	8	0	1	2	0
Is-1	Hap_36	1	0	0	0	0	0	0	1	0	0	0	0
Is-1	Hap_37	4	0	0	0	0	0	0	4	0	0	0	0
Is-1	Hap_38	4	0	0	0	4	0	0	0	0	0	0	0
Is-1	Hap_39	3	0	0	1	0	1	0	1	0	0	0	0
Is-1	Hap_40	1	0	0	0	0	1	0	0	0	0	0	0
Is-1	Hap_41	1	0	0	0	0	0	1	0	0	0	0	0
Is-1	Hap_42	1	0	0	0	0	0	0	1	0	0	0	0
Is-1	Hap_43	3	0	0	0	3	0	0	0	0	0	0	0
Is-1	Hap_44	1	0	0	0	0	0	0	0	0	0	1	0
Is-1	Hap_45	1	0	0	0	0	1	0	0	0	0	0	0
Is-1	Hap_47	1	0	0	0	0	0	1	0	0	0	0	0
Is-1	Hap_48	1	0	0	0	0	1	0	0	0	0	0	0
Is-1	Hap_49	1	0	0	0	0	0	0	1	0	0	0	0
Is-1	Hap_50	4	0	0	0	4	0	0	0	0	0	0	0
Is-1	Hap_51	5	0	0	0	5	0	0	0	0	0	0	0
Is-1	Hap_63	3	0	0	0	3	0	0	0	0	0	0	0
Is-1	Hap_79	2	1	0	0	1	0	0	0	0	0	0	0

Table 6.3. Continued.

16S haplotype:		*n*	No. individuals from:*										
3′	5′		AB	SK	MB	MN	ON	QC	RI	NB	PE	NS	NL
Is-1	Hap_80	1	0	0	0	1	0	0	0	0	0	0	0
Is-1	Hap_82	1	0	0	0	1	0	0	0	0	0	0	0
Is-1	Hap_83	1	0	0	0	1	0	0	0	0	0	0	0
Is-1	Hap_84	2	0	0	0	2	0	0	0	0	0	0	0
Is-1	Hap_86	2	0	0	2	0	0	0	0	0	0	0	0
Is-1	Hap_87	1	0	0	0	1	0	0	0	0	0	0	0
Is-1	Hap_88	1	0	0	0	1	0	0	0	0	0	0	0
Is-1	Hap_90	1	0	0	0	1	0	0	0	0	0	0	0
Is-1	Hap_91	1	0	0	0	1	0	0	0	0	0	0	0
Is-1	Hap_92	3	0	0	0	3	0	0	0	0	0	0	0
Is-1	Hap_93	2	0	0	0	2	0	0	0	0	0	0	0
Is-1	Hap_95	3	0	0	0	0	3	0	0	0	0	0	0
Is-1	Hap_96	1	0	0	0	0	1	0	0	0	0	0	0
Is-1	Hap_98	3	0	0	0	0	3	0	0	0	0	0	0
Is-1	Hap_100	1	0	0	0	0	1	0	0	0	0	0	0
Is-1	Hap_104	2	0	0	0	0	2	0	0	0	0	0	0
Is-1	Hap_105	1	0	0	0	0	1	0	0	0	0	0	0
Is-1	Hap_110	1	0	0	1	0	0	0	0	0	0	0	0
Is-1	Hap_115	1	0	0	1	0	0	0	0	0	0	0	0
Is-1	Hap_116	1	0	0	1	0	0	0	0	0	0	0	0
Is-1	Hap_118	1	0	0	0	1	0	0	0	0	0	0	0
Is-1	Hap_124	1	0	0	0	0	0	0	1	0	0	0	0
Is-1	Hap_125	1	0	0	0	1	0	0	0	0	0	0	0
Is-1	Hap_128	1	0	0	1	0	0	0	0	0	0	0	0
Is-2	Hap_1	8	0	0	0	0	0	1	6	1	0	0	0
Is-2	Hap_7	5	0	0	4	1	0	0	0	0	0	0	0
Is-2	Hap_54	1	0	0	0	0	0	0	1	0	0	0	0
Is-2	Hap_56	1	0	0	0	0	0	0	1	0	0	0	0
Is-2	Hap_57	2	0	0	0	0	2	0	0	0	0	0	0
Is-2	Hap_58	1	0	0	0	0	0	0	1	0	0	0	0
Is-2	Hap_60	1	0	0	0	0	0	0	1	0	0	0	0
Is-2	Hap_61	2	0	0	0	0	0	0	1	0	0	1	0
Is-2	Hap_63	1	0	0	0	0	0	0	1	0	0	0	0
Is-2	Hap_68	3	0	0	2	1	0	0	0	0	0	0	0
Is-3	Hap_1	1	0	0	0	0	0	1	0	0	0	0	0
Is-4	Hap_3	7	0	0	6	1	0	0	0	0	0	0	0
Is-4	Hap_53	2	0	0	2	0	0	0	0	0	0	0	0
Is-4	Hap_59	7	0	1	0	0	1	2	3	0	0	0	0
Is-4	Hap_62	9	0	0	0	0	0	1	6	0	1	0	1
Is-4	Hap_67	1	0	0	0	0	0	0	0	0	1	0	0
Is-4	Hap_69	1	0	0	0	1	0	0	0	0	0	0	0
Is-4	Hap_71	1	0	0	0	1	0	0	0	0	0	0	0
Is-4	Hap_72	10	0	0	4	6	0	0	0	0	0	0	0
Is-4	Hap_73	1	0	0	0	1	0	0	0	0	0	0	0
Is-4	Hap_74	2	0	0	1	1	0	0	0	0	0	0	0
Is-4	Hap_85	1	0	0	0	1	0	0	0	0	0	0	0
Is-4	Hap_89	1	0	0	1	0	0	0	0	0	0	0	0
Is-4	Hap_94	1	0	0	0	0	1	0	0	0	0	0	0
Is-5	Hap_59	3	0	0	0	0	0	0	3	0	0	0	0
Is-6	Hap_39	6	0	0	1	5	0	0	0	0	0	0	0

Table 6.3. Continued.

16S haplotype:		*n*	No. individuals from:*									
3′	5′		AB	SK	MB	MN	ON	QC	RI	NB	PE	NS	NL
Is-6	Hap_75	7	0	0	0	0	0	4	1	1	0	1	0
Is-6	Hap_99	1	0	0	0	0	1	0	0	0	0	0	0
Is-7	Hap_52	14	0	0	0	0	1	0	11	0	0	1	1
Is-7	Hap_55	1	0	0	0	0	0	0	1	0	0	0	0
Is-7	Hap_64	2	0	0	0	0	0	0	2	0	0	0	0
Is-7	Hap_66	10	0	0	0	0	1	0	9	0	0	0	0
Is-7	Hap_70	1	0	0	0	0	0	0	1	0	0	0	0
Is-7	Hap_77	1	0	0	0	0	0	0	0	1	0	0	0
Is-7	Hap_78	1	0	0	0	0	0	1	0	0	0	0	0
Is-7	Hap_96	3	0	0	0	0	3	0	0	0	0	0	0
Is-8	Hap_1	2	0	0	0	2	0	0	0	0	0	0	0
Is-8	Hap_7	1	0	0	1	0	0	0	0	0	0	0	0
Is-9	Hap_2	10	0	0	4	2	4	0	0	0	0	0	0
Is-9	Hap_76	1	0	0	0	1	0	0	0	0	0	0	0
Is-10	Hap_102	2	0	0	0	0	2	0	0	0	0	0	0
Is-11	Hap_130	1	0	0	0	0	1	0	0	0	0	0	0
Is-12	Hap_14	3	0	0	0	0	0	0	2	0	0	1	0
Is-13	Hap_1	1	0	1	0	0	0	0	0	0	0	0	0
Is-13	Hap_2	12	0	0	1	3	8	0	0	0	0	0	0
Is-13	Hap_5	1	0	0	0	1	0	0	0	0	0	0	0
Is-13	Hap_22	1	0	0	1	0	0	0	0	0	0	0	0
Is-13	Hap_45	1	0	0	0	1	0	0	0	0	0	0	0
Is-13	Hap_81	1	0	0	0	1	0	0	0	0	0	0	0
Is-13	Hap_83	1	0	0	0	1	0	0	0	0	0	0	0
Is-13	Hap_97	5	0	0	0	0	5	0	0	0	0	0	0
Is-13	Hap_103	1	0	0	0	0	1	0	0	0	0	0	0
Is-14	Hap_126	2	0	0	0	2	0	0	0	0	0	0	0
Is-14	Hap_127	1	0	0	0	1	0	0	0	0	0	0	0
Is-15	Hap_1	4	0	0	0	0	4	0	0	0	0	0	0
Is-15	Hap_45	22	0	0	0	0	22	0	0	0	0	0	0
Is-15	Hap_115	1	0	0	0	0	1	0	0	0	0	0	0
Is-17	Hap_111	1	0	0	0	1	0	0	0	0	0	0	0
Is-20	Hap_1	2	0	0	0	0	2	0	0	0	0	0	0
Is-20	Hap_120	2	0	0	0	0	0	0	0	1	0	1	0
Is-21	Hap_2	1	0	0	0	0	0	1	0	0	0	0	0
Is-23	Hap_119	2	0	0	1	1	0	0	0	0	0	0	0
Is-24	Hap_20	2	0	0	0	0	0	1	0	0	0	1	0
Is-30	Hap_85	2	0	0	2	0	0	0	0	0	0	0	0
Is-48	Hap_7	2	0	0	0	2	0	0	0	0	0	0	0
Is-49	Hap_113	1	0	0	0	0	0	0	1	0	0	0	0
Is-50	Hap_122	2	0	0	0	0	2	0	0	0	0	0	0
Is-50	Hap_123	1	0	0	0	0	1	0	0	0	0	0	0
Is-51	Hap_112	4	0	0	3	1	0	0	0	0	0	0	0
Is-52	Hap_76	4	0	0	2	2	0	0	0	0	0	0	0
Is-53	Hap_2	2	0	0	1	1	0	0	0	0	0	0	0
Is-54	Hap_6	1	0	0	1	0	0	0	0	0	0	0	0
Is-54	Hap_35	1	0	0	0	1	0	0	0	0	0	0	0
Is-54	Hap_45	1	0	0	0	1	0	0	0	0	0	0	0
Is-55	Hap_2	1	0	0	0	1	0	0	0	0	0	0	0
Is-55	Hap_83	8	0	0	0	8	0	0	0	0	0	0	0

Table 6.3. Continued.

16S haplotype:		*n*	No. individuals from:*									
3′	5′		AB	SK	MB	MN	ON	QC	RI	NB	PE	NS	NL
Is-56	Hap_2	2	0	0	0	2	0	0	0	0	0	0	0
Is-57	Hap_117	6	0	0	5	1	0	0	0	0	0	0	0
Is-58	Hap_87	1	0	1	0	0	0	0	0	0	0	0	0
Is-59	Hap_109	2	0	0	0	2	0	0	0	0	0	0	0
Is-60	Hap_2	2	0	0	0	2	0	0	0	0	0	0	0
Is-61	Hap_2	1	0	0	0	1	0	0	0	0	0	0	0
Is-62	Hap_2	4	0	0	0	4	0	0	0	0	0	0	0
Is-63	Hap_2	3	0	0	0	0	1	1	1	0	0	0	0
Is-63	Hap_35	3	0	0	0	0	0	1	2	0	0	0	0
Is-63	Hap_45	13	1	0	0	0	10	0	1	1	0	0	0
Is-63	Hap_46	1	0	0	0	0	0	0	1	0	0	0	0
Is-64	Hap_107	1	0	0	0	1	0	0	0	0	0	0	0
Is-65	Hap_34	1	0	0	0	1	0	0	0	0	0	0	0
Is-66	Hap_2	1	0	0	0	1	0	0	0	0	0	0	0
Is-67	Hap_20	1	0	0	1	0	0	0	0	0	0	0	0
Is-68	Hap_45	1	0	0	1	0	0	0	0	0	0	0	0
Is-69	Hap_35	2	0	0	2	0	0	0	0	0	0	0	0
Is-69	Hap_110	1	0	0	1	0	0	0	0	0	0	0	0
Is-70	Hap_72	2	0	0	2	0	0	0	0	0	0	0	0
Is-71	Hap_106	1	0	1	0	0	0	0	0	0	0	0	0
Is-72	Hap_110	2	0	0	0	0	2	0	0	0	0	0	0
Is-73	Hap_121	2	0	0	0	0	2	0	0	0	0	0	0
Is-74	Hap_65	1	0	0	0	0	1	0	0	0	0	0	0
Is-74	Hap_129	5	0	0	0	0	5	0	0	0	0	0	0
Is-75	Hap_45	1	0	0	0	0	1	0	0	0	0	0	0
Is-76	Hap_101	2	0	0	0	0	2	0	0	0	0	0	0
Is-77	Hap_114	2	0	0	0	0	0	0	1	1	0	0	0
Is-78	Hap_2	1	0	0	0	0	1	0	0	0	0	0	0
Is-79	Hap_108	1	0	0	0	0	0	0	1	0	0	0	0
Total		576	2	6	95	167	163	22	98	6	3	12	2

* AB = Alberta, SK = Saskatchewan, MB = Manitoba, MN = Minnesota, ON = Ontario, QC = Quebec, RI = Rhode Island, NB = New Brunswick, PE = Prince Edward Island, NS = Nova Scotia, and NL = Newfoundland.

The remaining 172 haplotypes each represented less than 2% of the *I. scapularis*. Although the majority of the haplotypes were detected in one locality only, five haplotypes (i.e., Is-4 + Hap_59, Is-4 + Hap_62, Is-6 + Hap_75, Is-7 + Hap_52, and Is-63 + Hap_45) were each present in four localities and three haplotypes (i.e., Is-1 + Hap_2, Is-1 + Hap_22, and Is-1 + Hap_34) were each present in five localities. A total of 91 haplotypes (i.e., 50.8% of the 179 haplotypes) were each represented by a single tick.

The concatenated sequence data (1126 bp total) of the mt 12S rRNA + $tRNA^{Val}$ genes (Chapter 3), as well as Domains I and II (this chapter), and IV and V (Chapter 2 and Krakowetz et al. 2011) of the mt 16S rRNA gene showed that there were 117 haplotypes among the 225 *I. scapularis* that were characterized using all three DNA fragments (Table 6.4). These 117 concatenated haplotypes were comprised of 53 of the 56 haplotypes of Domains IV and V of the mt 16S rRNA gene that were detected in Chapter 2 and by Krakowetz et al. (2011), 61 of the 62 haplotypes of the mt 12S rRNA + $tRNA^{Val}$ genes that were detected in Chapter 3, as well as 75 of the 130 haplotypes of Domains I and II of the mt 16S rRNA gene that were detected in the present study. The most common haplotype was Is-15 + CT07 + Hap_45, which comprised 9.3% of the 225 *I. scapularis* characterized using the three DNA fragments. The second most common haplotype (i.e., Is-1 + CT08 + Hap_2) represented 7.6% of the ticks. The remaining 115 haplotypes each represented less than 3% of the *I. scapularis*. Although most haplotypes were present in one locality only and eight haplotypes (i.e., Is-1 + CT45 + Hap_34, Is-12 + CT06 + Hap_14, Is-20 + CT37 + Hap_120, Is-23 + CT24 + Hap_119, Is-51 + CT23 + Hap_112, Is-52 + CT11 + Hap_76, Is-53 + CT08 + Hap_2, Is-57 + CT28 + Hap_117) were detected in two localities, only one haplotype (i.e., Is-1 + CT08 + Hap_2) was present in three different localities. Of the 117 concatenated haplotypes, 73 (i.e., 62.4%) were each represented by a single *I. scapularis*.

Eighty ticks of the 282 *I. scapularis* previously characterized as haplotype Is-1 in Chapter 2 corresponded to 30 haplotypes of the mt 12S rRNA + $tRNA^{Val}$ genes (Chapter 3) and 40 haplotypes of Domains I and II of the mt 16S rRNA gene (present chapter) for a total of 46 concatenated haplotypes (Table 6.4). The remaining 145 ticks representing the other 52 haplotypes of Domains IV and V of the mt 16S rRNA gene included in this study corresponded to 31 and 35 haplotypes of the mt 12S rRNA + $tRNA^{Val}$ genes and Domains I and II of the mt 16S rRNA gene, respectively, for a total of 71 concatenated haplotypes.

Table 6.4. The number of *I. scapularis* individuals of each haplotype based on the concatenated data for the 3' (Is series) and 5' (Hap series) ends of the mt 16S rRNA gene, as well as the mt 12S rRNA + $tRNA^{Val}$ genes (CT series). The haplotype based on the 3' end of the mt 16S rRNA gene of each tick was determined in Chapter 2 or by Krakowetz et al. (2011). The haplotype based on the mt 12S rRNA + $tRNA^{Val}$ genes of each tick was determined in Chapter 3.

Haplotype:			*n*	No. individuals from:*							
16S 3'	12S	16S 5'		SK	MB	MN	ON	QC	RI	NB	NS
Is-1	CT03	Hap_21	2	0	0	2	0	0	0	0	0
Is-1	CT05	Hap_38	3	0	0	3	0	0	0	0	0
Is-1	CT06	Hap_1	2	0	0	0	2	0	0	0	0
Is-1	CT06	Hap_63	2	0	0	2	0	0	0	0	0
Is-1	CT06	Hap_116	1	0	1	0	0	0	0	0	0
Is-1	CT07	Hap_45	1	0	0	0	1	0	0	0	0
Is-1	CT08	Hap_2	17	0	4	6	7	0	0	0	0
Is-1	CT08	Hap_7	1	0	0	1	0	0	0	0	0
Is-1	CT08	Hap_17	1	0	0	1	0	0	0	0	0
Is-1	CT08	Hap_50	2	0	0	2	0	0	0	0	0
Is-1	CT08	Hap_51	1	0	0	1	0	0	0	0	0
Is-1	CT08	Hap_86	2	0	2	0	0	0	0	0	0
Is-1	CT08	Hap_90	1	0	0	1	0	0	0	0	0
Is-1	CT08	Hap_93	2	0	0	2	0	0	0	0	0
Is-1	CT08	Hap_95	3	0	0	0	3	0	0	0	0
Is-1	CT08	Hap_105	1	0	0	0	1	0	0	0	0
Is-1	CT08	Hap_115	1	0	1	0	0	0	0	0	0
Is-1	CT08	Hap_124	1	0	0	0	0	0	1	0	0
Is-1	CT09	Hap_118	1	0	0	1	0	0	0	0	0
Is-1	CT10	Hap_29	2	0	0	2	0	0	0	0	0
Is-1	CT15	Hap_16	1	0	1	0	0	0	0	0	0
Is-1	CT19	Hap_128	1	0	1	0	0	0	0	0	0
Is-1	CT20	Hap_43	2	0	0	2	0	0	0	0	0
Is-1	CT21	Hap_27	1	0	1	0	0	0	0	0	0
Is-1	CT25	Hap_1	1	0	0	0	1	0	0	0	0
Is-1	CT25	Hap_26	4	0	0	0	4	0	0	0	0
Is-1	CT25	Hap_100	1	0	0	0	1	0	0	0	0
Is-1	CT27	Hap_83	1	0	0	1	0	0	0	0	0
Is-1	CT30	Hap_92	3	0	0	3	0	0	0	0	0
Is-1	CT32	Hap_2	1	0	0	1	0	0	0	0	0
Is-1	CT33	Hap_110	1	0	1	0	0	0	0	0	0
Is-1	CT44	Hap_91	1	0	0	1	0	0	0	0	0
Is-1	CT45	Hap_34	2	0	0	0	0	1	1	0	0
Is-1	CT47	Hap_87	1	0	0	1	0	0	0	0	0
Is-1	CT49	Hap_125	1	0	0	1	0	0	0	0	0
Is-1	CT50	Hap_80	1	0	0	1	0	0	0	0	0
Is-1	CT52	Hap_84	1	0	0	1	0	0	0	0	0
Is-1	CT53	Hap_2	1	0	0	1	0	0	0	0	0
Is-1	CT54	Hap_37	1	0	0	0	0	0	1	0	0
Is-1	CT55	Hap_98	1	0	0	0	1	0	0	0	0
Is-1	CT56	Hap_13	1	0	0	1	0	0	0	0	0
Is-1	CT56	Hap_88	1	0	0	1	0	0	0	0	0
Is-1	CT57	Hap_2	1	0	0	1	0	0	0	0	0
Is-1	CT58	Hap_7	1	0	1	0	0	0	0	0	0
Is-1	CT59	Hap_104	1	0	0	0	1	0	0	0	0
Is-1	CT62	Hap_98	1	0	0	0	1	0	0	0	0

Table 6.4. Continued.

Haplotype:			*n*	No. individuals from:*							
16S 3′	12S	16S 5′		SK	MB	MN	ON	QC	RI	NB	NS
Is-2	CT22	Hap_57	1	0	0	0	1	0	0	0	0
Is-3	CT46	Hap_1	1	0	0	0	0	1	0	0	0
Is-4	CT29	Hap_59	1	1	0	0	0	0	0	0	0
Is-4	CT29	Hap_89	1	0	1	0	0	0	0	0	0
Is-5	CT34	Hap_59	3	0	0	0	0	0	3	0	0
Is-6	CT08	Hap_99	1	0	0	0	1	0	0	0	0
Is-7	CT40	Hap_96	1	0	0	0	1	0	0	0	0
Is-8	CT12	Hap_7	1	0	1	0	0	0	0	0	0
Is-8	CT13	Hap_1	2	0	0	2	0	0	0	0	0
Is-9	CT08	Hap_2	1	0	0	1	0	0	0	0	0
Is-10	CT01	Hap_102	2	0	0	0	2	0	0	0	0
Is-11	CT04	Hap_130	1	0	0	0	1	0	0	0	0
Is-12	CT06	Hap_14	3	0	0	0	0	0	2	0	1
Is-13	CT05	Hap_103	1	0	0	0	1	0	0	0	0
Is-13	CT08	Hap_97	3	0	0	0	3	0	0	0	0
Is-13	CT41	Hap_2	1	0	1	0	0	0	0	0	0
Is-14	CT43	Hap_126	2	0	0	2	0	0	0	0	0
Is-14	CT43	Hap_127	1	0	0	1	0	0	0	0	0
Is-15	CT06	Hap_1	4	0	0	0	4	0	0	0	0
Is-15	CT07	Hap_45	21	0	0	0	21	0	0	0	0
Is-15	CT08	Hap_115	1	0	0	0	1	0	0	0	0
Is-17	CT07	Hap_111	1	0	0	1	0	0	0	0	0
Is-20	CT14	Hap_1	2	0	0	0	2	0	0	0	0
Is-20	CT37	Hap_120	2	0	0	0	0	0	0	1	1
Is-21	CT51	Hap_2	1	0	0	0	0	1	0	0	0
Is-23	CT24	Hap_119	2	0	1	1	0	0	0	0	0
Is-24	CT36	Hap_20	1	0	0	0	0	1	0	0	0
Is-24	CT42	Hap_20	1	0	0	0	0	0	0	0	1
Is-30	CT29	Hap_85	2	0	2	0	0	0	0	0	0
Is-48	CT08	Hap_7	2	0	0	2	0	0	0	0	0
Is-49	CT40	Hap_113	1	0	0	0	0	0	1	0	0
Is-50	CT21	Hap_122	2	0	0	0	2	0	0	0	0
Is-50	CT23	Hap_123	1	0	0	0	1	0	0	0	0
Is-51	CT23	Hap_112	4	0	3	1	0	0	0	0	0
Is-52	CT11	Hap_76	4	0	2	2	0	0	0	0	0
Is-53	CT08	Hap_2	2	0	1	1	0	0	0	0	0
Is-54	CT07	Hap_45	1	0	0	1	0	0	0	0	0
Is-54	CT07	Hap_35	1	0	0	1	0	0	0	0	0
Is-54	CT17	Hap_6	1	0	1	0	0	0	0	0	0
Is-55	CT08	Hap_2	1	0	0	1	0	0	0	0	0
Is-55	CT27	Hap_83	5	0	0	5	0	0	0	0	0
Is-55	CT31	Hap_83	3	0	0	3	0	0	0	0	0
Is-56	CT08	Hap_2	2	0	0	2	0	0	0	0	0
Is-57	CT28	Hap_117	6	0	5	1	0	0	0	0	0
Is-58	CT47	Hap_87	1	1	0	0	0	0	0	0	0
Is-59	CT08	Hap_109	1	0	0	1	0	0	0	0	0
Is-59	CT48	Hap_109	1	0	0	1	0	0	0	0	0
Is-60	CT08	Hap_2	2	0	0	2	0	0	0	0	0
Is-61	CT56	Hap_2	1	0	0	1	0	0	0	0	0
Is-62	CT08	Hap_2	4	0	0	4	0	0	0	0	0

Table 6.4. Continued.

Haplotype: 16S 3′	12S	16S 5′	*n*	No. individuals from:* SK	MB	MN	ON	QC	RI	NB	NS
Is-63	CT07	Hap_45	4	0	0	0	4	0	0	0	0
Is-63	CT08	Hap_2	1	0	0	0	1	0	0	0	0
Is-64	CT25	Hap_107	1	0	0	1	0	0	0	0	0
Is-65	CT45	Hap_34	1	0	0	1	0	0	0	0	0
Is-66	CT60	Hap_2	1	0	0	1	0	0	0	0	0
Is-67	CT32	Hap_20	1	0	1	0	0	0	0	0	0
Is-68	CT07	Hap_45	1	0	1	0	0	0	0	0	0
Is-69	CT07	Hap_35	2	0	2	0	0	0	0	0	0
Is-69	CT18	Hap_110	1	0	1	0	0	0	0	0	0
Is-70	CT29	Hap_72	2	0	2	0	0	0	0	0	0
Is-71	CT26	Hap_106	1	1	0	0	0	0	0	0	0
Is-72	CT08	Hap_110	2	0	0	0	2	0	0	0	0
Is-73	CT16	Hap_121	2	0	0	0	2	0	0	0	0
Is-74	CT02	Hap_65	1	0	0	0	1	0	0	0	0
Is-74	CT02	Hap_129	5	0	0	0	5	0	0	0	0
Is-75	CT07	Hap_45	1	0	0	0	1	0	0	0	0
Is-76	CT38	Hap_101	3	0	0	0	3	0	0	0	0
Is-77	CT35	Hap_114	1	0	0	0	0	0	0	1	0
Is-77	CT39	Hap_114	1	0	0	0	0	0	1	0	0
Is-78	CT08	Hap_2	1	0	0	0	1	0	0	0	0
Is-79	CT40	Hap_108	1	0	0	0	0	0	1	0	0
Total			225	3	38	80	84	4	11	2	3

* SK = Saskatchewan, MB = Manitoba, MN = Minnesota, ON = Ontario, QC = Quebec, RI = Rhode Island, NB = New Brunswick, and NS = Nova Scotia.

6.4.2.2. Phylogeographical analyses

The minimum spanning network depicting the relationships among the concatenated haplotypes (i.e., Domains I and II + IV and V of the mt 16S rRNA gene) showed a combination of "star," "starburst," and "chain" patterns (Fig. 6.8). The most frequently detected haplotype (i.e., Is-1 + Hap_2) represented the central haplotype in the "star" pattern from which 28 (i.e., 15.6%) of the 179 haplotypes directly differed by 1 or 2 bp. Some of the numerous "starburst" patterns were centered on haplotypes Is-1 + Hap_7, Is-1 + Hap_1, Is-1 + Hap_110, Is-2 + Hap_1, Is-7 + Hap_52, Is-1 + Hap_39, Is-1 + Hap_20, and Is-4 + Hap_72. Only four (i.e., Is-1 + Hap_2, Is-7 + Hap_52, Is-15 + Hap_45, and Is-63 + Hap_45) of the seven most commonly detected haplotypes in the present study represented links to four or more real (i.e., detected) or putative haplotypes (Table 6.3 and Fig. 6.8). The other three common haplotypes were linked to two (i.e., Is-1 + Hap_26) or three (i.e., Is-1 + Hap_34 and Is-13 + Hap_2) real or putative haplotypes. One "chain" was comprised of haplotypes Is-54 + Hap_45, Is-54 + Hap_35, and Is-54 + Hap_6, whereas the other included haplotypes Is-63 + Hap_2, Is-13 + Hap_5, and Is-13 + Hap_83.

The seven most commonly detected haplotypes occurred in "eastern" localities only or in both "eastern" and "western" localities, but never in "western" localities only (Table 6.3 and Fig. 6.8). Of the 179 total haplotypes detected within the entire sample set of 576 *I. scapularis* individuals, 99 (i.e., 55.3%) haplotypes were represented by 212 ticks collected from the "western" geographical region only (i.e., 36.8% of the 576 ticks collected in total), whereas only 72 (i.e., 40.2%) haplotypes corresponded to 215 ticks collected from the "eastern" geographical region only (i.e., 37.3% of the 576 ticks). The eight remaining haplotypes (i.e., 4.5%) representing a total of 149 or 25.9% of the 576 ticks were each derived from *I. scapularis* collected from the "eastern" and "western" geographical areas.

There were several relatively distinct phylogroups (i.e., phylogroups a-g) that corresponded to haplotypes of the "eastern" geographical region (Fig. 6.8). One phylogroup (i.e., phylogroup f) was particularly distinct (i.e., ≥ 5 bp different) from the other haplotypes in the network, including its neighbouring phylogroup (i.e., phylogroup e). Phylogroups c, d, e, and g may be interconnected and appear to be distinct from phylogroups a and b, which appear to be genetically distinct from each other. Haplotypes of the "eastern" phylogroups were not associated with particular geographical areas or tick populations on a local scale (see Table 6.3 and Fig. 6.8).

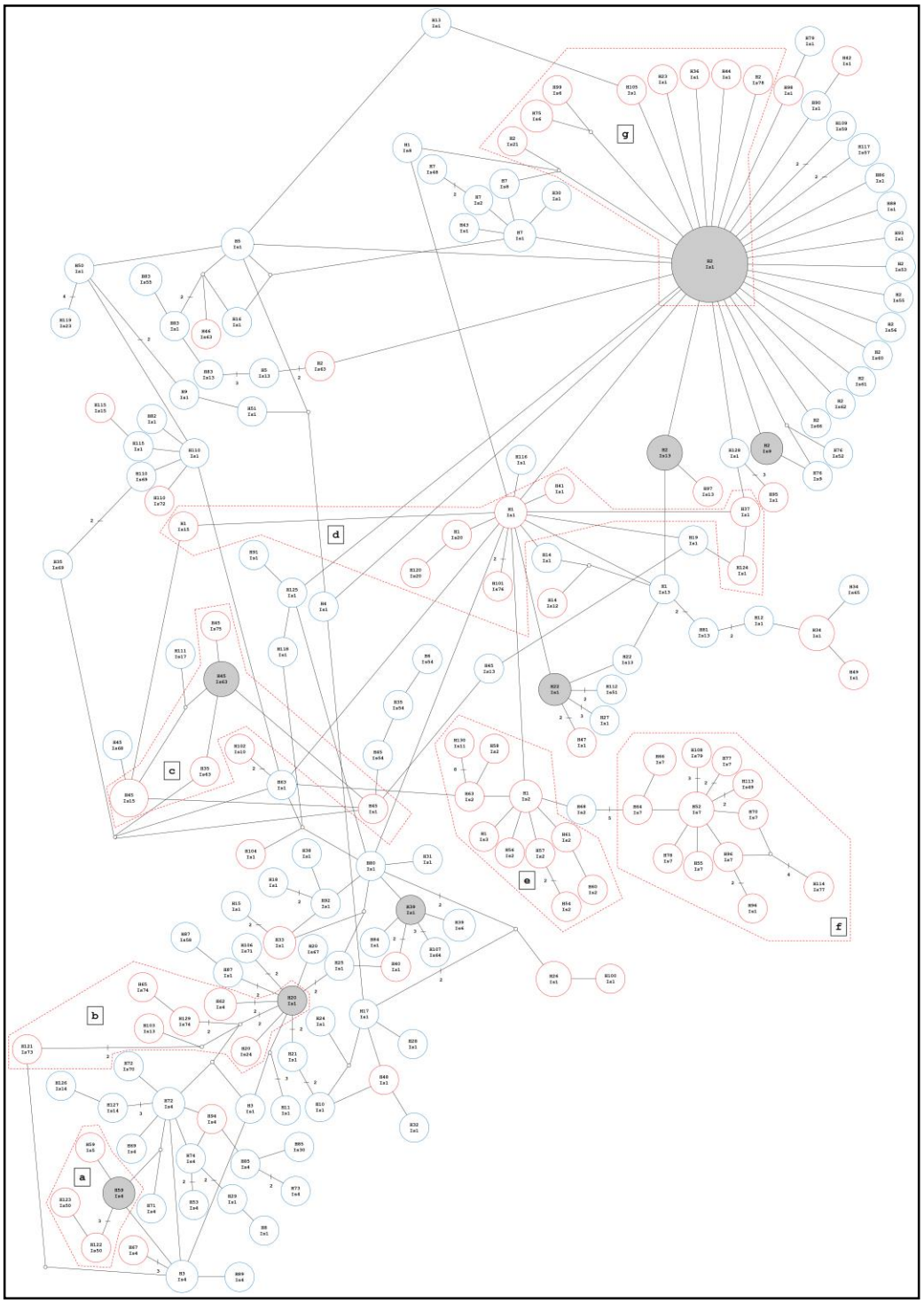


Figure 6.8. A minimum spanning network depicting the relationships among haplotypes of Domains I and II + Domains IV and V of the mt 16S rRNA gene of *I. scapularis*. Numerical designations, which correspond to those in Table 6.3, have been assigned to the 179 haplotypes. The number of nucleotide differences between pairs of DNA sequences (circles) is one unless otherwise indicated. The size of each circle is proportional to the number of ticks of that haplotype. Putative haplotypes are represented by small open circles. Haplotypes are denoted by blue, grey, or red circles depending on whether each was detected in the "western," "western" and "eastern," or "eastern" geographical areas, respectively. Several relatively distinct phylogroups (a-g), each of which is comprised of haplotypes corresponding to the "eastern" geographical region, are contained within the red polygons.

Fig. 6.9 shows that the identities and abundances of the most frequently detected haplotypes based on the three DNA fragments corresponding to the mt 12S rRNA + $tRNA^{Val}$ genes (Chapter 3), as well as Domains I and II (this chapter) and IV and V (Chapter 2 and Krakowetz et al. 2011) of the mt 16S rRNA gene, differed among the nine established populations. There were thirteen shared haplotypes among the established populations of *I. scapularis* (Fig. 6.10); however, only one haplotype (i.e., Is-1 + CT08 + Hap_2) was represented by two or more ticks across multiple (i.e., five) populations (Fig. 6.9). The remaining twelve haplotypes that occurred in two or more of the established populations were each represented by a single tick in one population, as well as by two or more *I. scapularis* in another population, or, in one case (i.e., Is-52 + CT11 + Hap_76), by a single tick in two populations and by ≥ 2 ticks in another population. The number of haplotypes detected in CR, St. Croix State Park (CSP), and LPPP (i.e., three of the nine established populations where samples sizes exceeded 30 ticks) was approximately equal at 26 haplotypes (n = 36), 20 haplotypes (n = 31), and 25 haplotypes (n = 66), respectively (Fig. 6.10). However, there was nearly twice as many haplotypes detected per tick sampled in CR and CSP in the west as compared to LPPP in the east.

The identities and abundances of the most frequently detected haplotypes (i.e., mt 12S rRNA and $tRNA^{Val}$ genes + Domains I and II + IV and V of the mt 16S rRNA gene) also differed among the eight provinces and states (Table 6.4). The number of shared haplotypes among the *I. scapularis* in the different provinces and states was fewer than that among the established populations at the nine localities (Fig. 6.11). A single haplotype (i.e., Is-1 + CT08 + Hap_2) was detected in three provinces/states, whereas the other eight haplotypes were shared between two of them. Five of these nine shared haplotypes were present in "western" provinces/states only, while three were detected in "eastern" provinces/states only. Haplotype Is-1 + CT08 + Hap_2 represented ticks from both the "western" and "eastern" geographical regions. An approximately equal number of ticks from MN and ON was characterized using the three DNA fragments; however, 50% more haplotypes were detected in MN in the "western" geographical region as compared to ON in the "eastern" geographical region.

Fig. 6.12 depicts the relationships among the 117 concatenated haplotypes of the three DNA fragments corresponding to the mt 12S rRNA + $tRNA^{Val}$ genes (Chapter 3), as well as

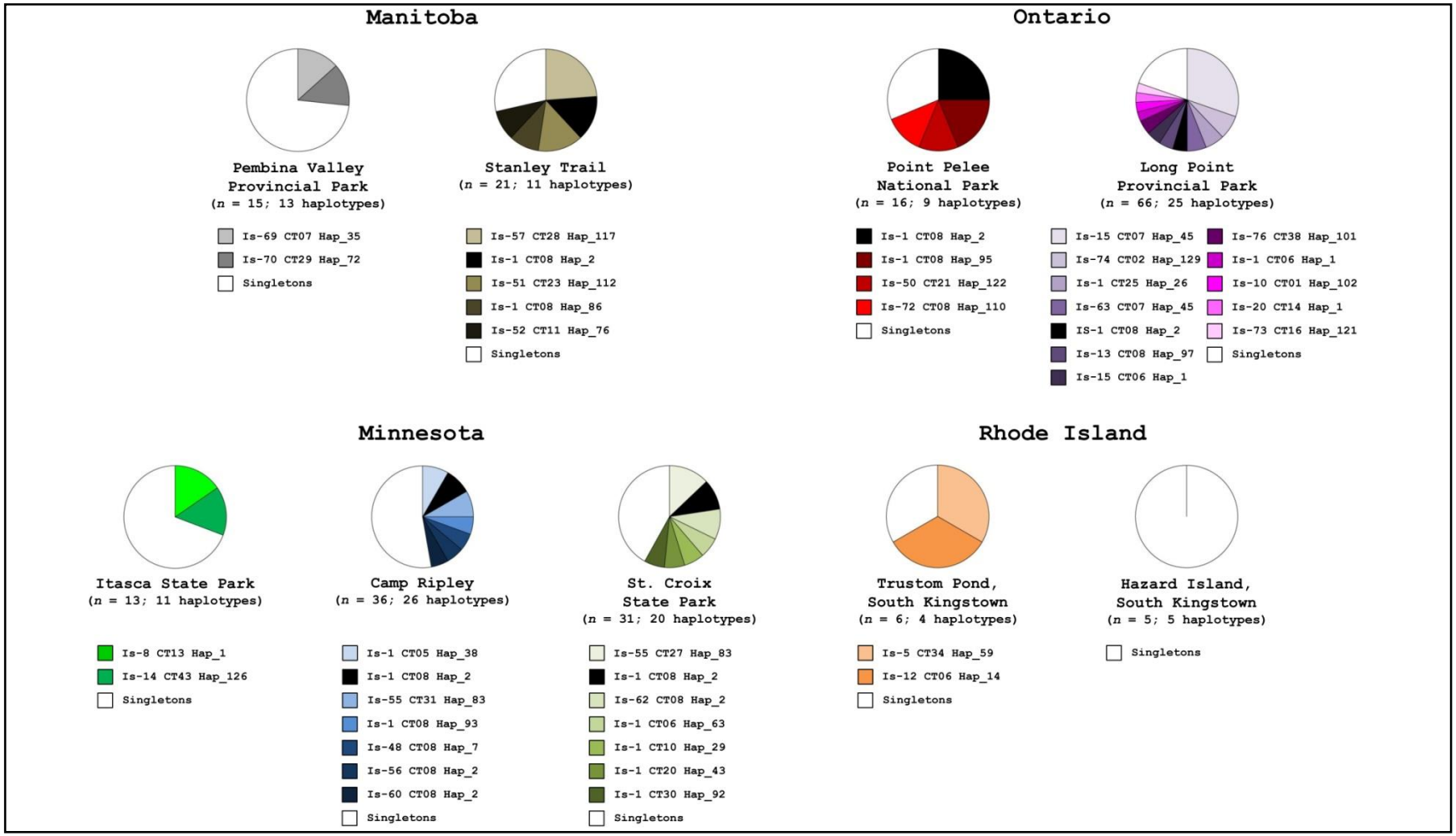


Figure 6.9. Diagram representing the identities and abundances of the most common haplotypes detected within each of the nine established populations of *I. scapularis* (n = 209), based on the three concatenated DNA fragments (i.e., Domains I and II + Domains IV and V of the mt 16S rRNA gene, as well as the mt 12S rRNA + $tRNA^{Val}$ genes). The number of haplotypes that were found in each population (in parentheses), as well as the sample size (n) corresponding to each population are also reported. Manitoba and Ontario are provinces in Canada, while Minnesota and Rhode Island are states in the U.S.A.

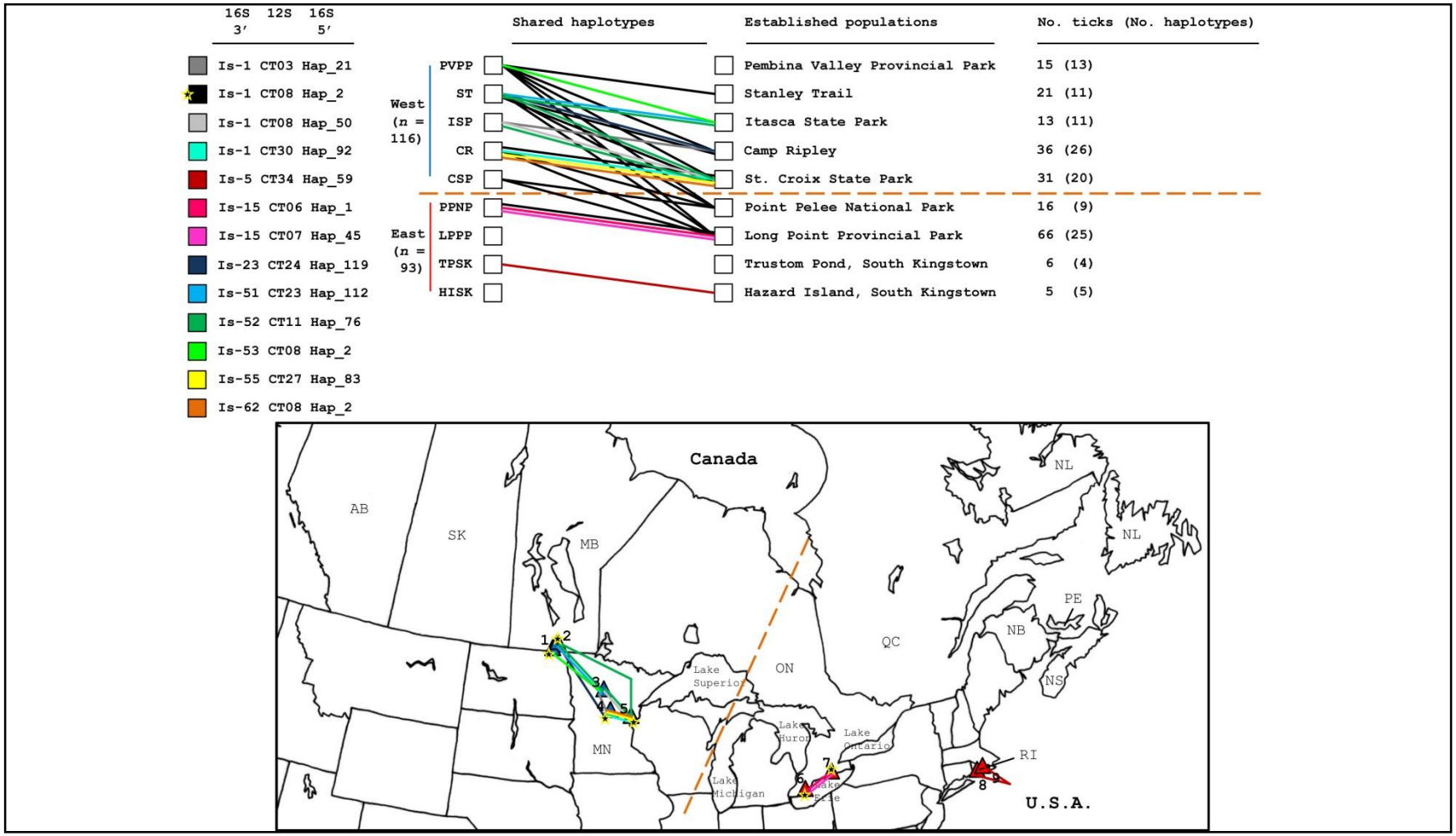


Figure 6.10. Shared haplotypes between pairs of established populations of *I. scapularis* ($n = 209$), based on the three concatenated DNA fragments (i.e., Domains I and II (16S 5') + Domains IV and V (16S 3') of the mt 16S rRNA gene, as well as the mt 12S rRNA + $tRNA^{Val}$ (12S) genes). The number of haplotypes that were found in each population (in parentheses), as well as the sample size (no. ticks) corresponding to each population are also reported. Populations in: Manitoba, Canada = (1) PVPP and (2) ST; Minnesota, U.S.A. = (3) ISP, (4) CR, and (5) CSP; Ontario, Canada = (6) PPNP and (7) LPPP; and Rhode Island, U.S.A. = (8) TPSK and (9) HISK. The colour corresponding to a particular haplotype also corresponds to the colour of a line(s) between pairs of populations. The "western" and "eastern" geographical regions are separated by the orange dashed line.

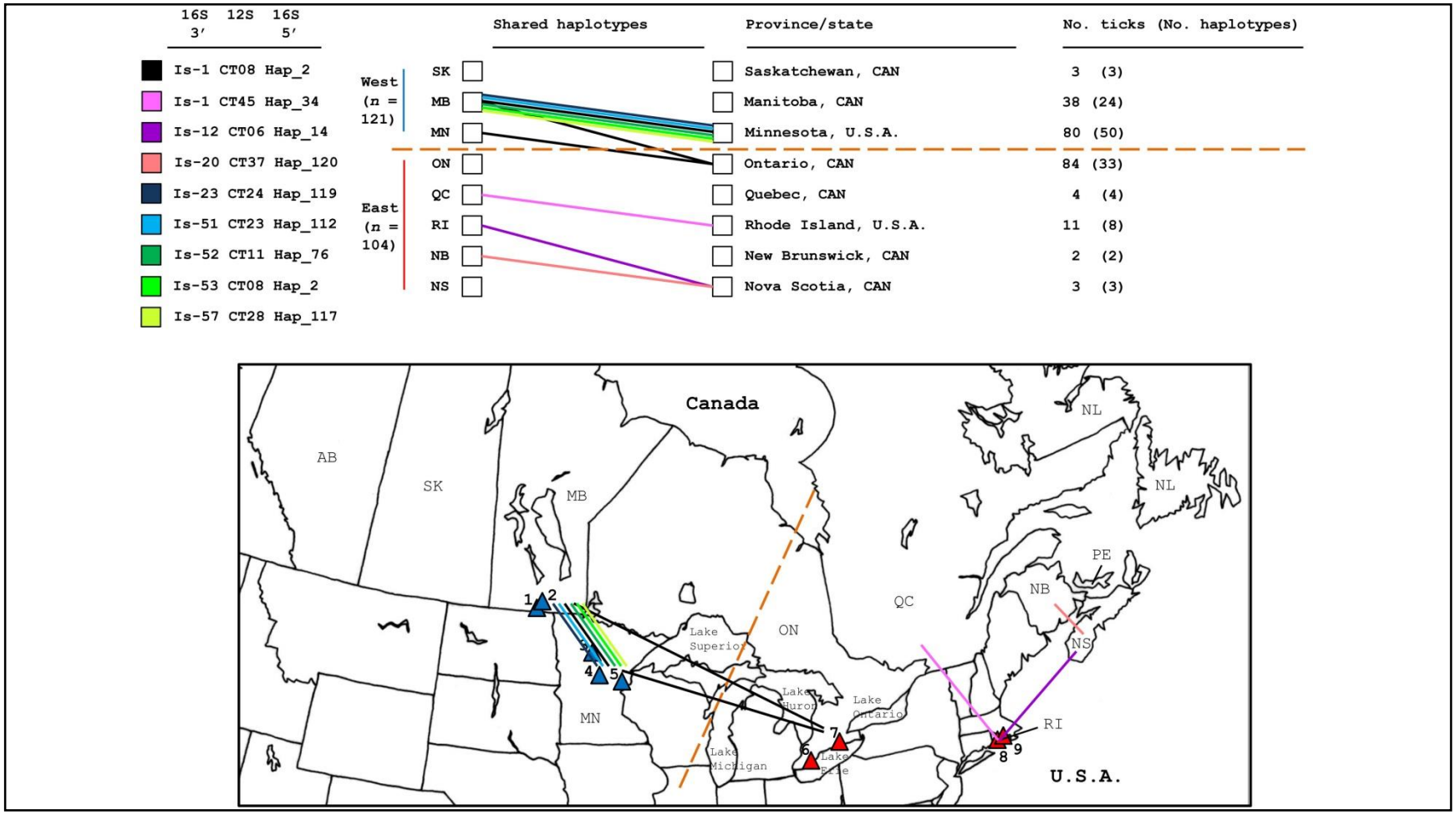


Figure 6.11. Shared haplotypes, based on the three concatenated DNA fragments (i.e., Domains I and II (16S 5') + Domains IV and V (16S 3') of the mt 16S rRNA gene, as well as the mt 12S rRNA + $tRNA^{Val}$ (12S) genes), between pairs of localities (provinces and/or states) in which *I. scapularis* ($n = 225$) were collected. The number of haplotypes that were found in each locality (in parentheses), as well as the sample size (no. ticks) corresponding to each locality are also reported. Populations in: Manitoba, Canada = (1) Pembina Valley Provincial Park and (2) Stanley Trail; Minnesota, U.S.A. = (3) Itasca State Park, (4) Camp Ripley, and (5) St. Croix State Park; Ontario, Canada = (6) Point Pelee National Park and (7) Long Point Provincial Park; and Rhode Island, U.S.A. = (8) Trustom Pond, South Kingstown and (9) Hazard Island, South Kingstown. The colour corresponding to a particular haplotype also corresponds to the colour of a line(s) between pairs of localities. The "western" and "eastern" geographical regions are separated by the orange dashed line.

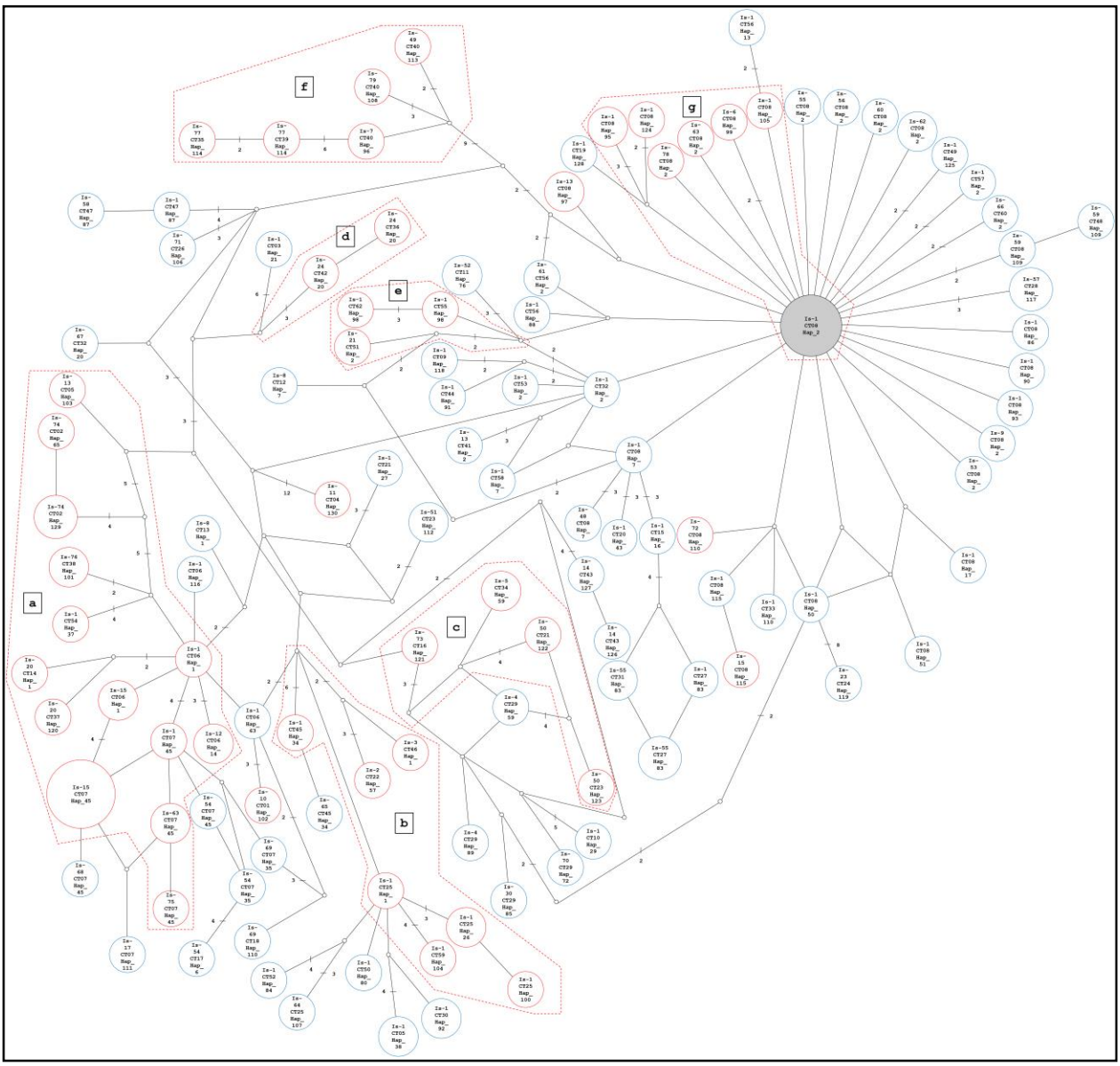


Figure 6.12. A minimum spanning network depicting the relationships among the concatenated haplotypes of Domains I and II + Domains IV and V of the mt 16S rRNA gene, as well the mt 12S rRNA + tRNA^{Val} genes of *I. scapularis*. Numerical designations, which correspond to those in Table 6.4, have been assigned to the 117 haplotypes. The number of nucleotide differences between pairs of DNA sequences (circles) is one unless otherwise indicated. The size of each circle is proportional to the number of ticks of that haplotype. Putative haplotypes are represented by small open circles. Haplotypes are denoted by blue, grey, or red circles depending on whether each was detected in the "western," "western" and "eastern," or "eastern" geographical areas, respectively. Several relatively distinct phylogroups (a-g), each of which is comprised of haplotypes corresponding to the "eastern" geographical region, are contained within the red polygons.

Domains I and II (this chapter) and IV and V (Chapter 2 and Krakowetz et al. 2011) of the mt 16S rRNA gene. The overall pattern of the minimum spanning network was a "star" phylogeny; however, "starburst" and "chain" patterns were also present. The central haplotype of the "star" configuration was haplotype Is-1 + CT08 + Hap_2 from which 20 haplotypes directly differed by 1-3 bp. Every one of the 20 haplotypes directly linked to haplotype Is-1 + CT08 + Hap_2 were associated with ticks collected from MB, MN, or ON only (Table 6.4 and Fig. 6.12). In contrast, haplotypes representing ticks from Rhode Island differed from haplotype Is-1 + CT08 + Hap_2 by 3-20 bp, but mostly by ≥ 8 bp (Fig. 6.12). Although there were several "starburst" configurations present within the haplotype network, the nodes (i.e., central haplotypes) from which multiple haplotypes radiated from were generally putative rather than real haplotypes. For example, a putative haplotype, which directly differed from haplotype Is-1 + CT08 + Hap_2 by 1 bp, gave rise to haplotypes Is-1 + CT19 + Hap_128, Is-1 + CT08 + Hap_95, and Is-1 + CT08 + Hap_124. Some of the real haplotypes that represented "central" haplotypes of the "starburst" pattern were haplotypes Is-1 + CT32 + Hap_2 and Is-1 + CT08 + Hap_7, which differed from haplotype Is-1 + CT08 + Hap_2 by 1 bp, and haplotypes Is-15 + CT07 + Hap_45, Is-1 + CT07 + Hap_45, and Is-1 + CT06 + Hap_1, which were clustered together and differed from haplotype Is-1 + CT08 + Hap_2 by ≥ 6 bp. A "chain" pattern was represented by haplotypes Is-7 + CT40 + Hap_96, Is-77 + CT39 + Hap_114, and Is-77 + CT35 + Hap_114. Remarkably, the most frequently detected haplotype (i.e., Is-15 + CT07 + Hap_45) was not the "central" haplotype of the "star" configuration. Instead, the most common haplotype, which was detected in ON only, represented the central haplotype of a "starburst" pattern and differed from the "central" haplotype of the "star" phylogeny (i.e., Is-1 + CT08 + Hap_2) by 11 bp. The second most frequently detected haplotype (i.e., Is-1 + CT08 + Hap_2) represented ticks collected from MB, MN, and ON (i.e., both the "western" and "eastern" geographical regions).

Of the 117 haplotypes detected within the entire sample set of 225 ticks, 70 (i.e., 59.8%) haplotypes corresponded to 111 *I. scapularis* collected from the "western" geographical region only (i.e., 49.3% of the 225 ticks), whereas only 46 (i.e., 39.3%) haplotypes were represented by 97 ticks collected from "eastern" localities only (i.e., 43.1% of the 225 ticks) (Table 6.4 and Fig. 6.12). A single haplotype Is-1 + CT08 + Hap_2 was represented by 17 ticks (i.e., 7.6% of the 225 ticks) that were collected from the "western" and "eastern" geographical areas. In general, haplotypes detected in the "eastern" geographical region gave rise to haplotypes found in the

"western" geographical area; however, the reverse of this pattern was also present (e.g., Is-1 + CT08 + Hap_115 to Is-15 + CT08 + Hap_115 and Is-1 + CT06 + Hap_63 to Is-10 + CT01 + Hap_102). A total of seven relatively distinct phylogroups (i.e., phylogroups a-g) corresponded to haplotypes of the "eastern" geographical region only (Fig. 6.12). Phylogroup f was particularly distinct from the other haplotypes in the network (i.e., differed from its nearest neighbouring haplotype by 13 bp), as well as from the central haplotype of the "star" phylogeny (i.e., haplotype Is-1 + CT08 + Hap_2). Also, this phylogroup was primarily comprised of haplotypes of *I. scapularis* collected from Rhode Island. The largest phylogroup (i.e., phylogroup a) was comprised of 14 "eastern" haplotypes and differed from the central haplotype of the "star" phylogeny (i.e., haplotype Is-1 + CT08 + Hap_2) by 6 bp. Eleven of the fourteen haplotypes in phylogroup a represented ticks that had been collected from ON. There appears to be an association between some tick haplotypes and the geographical region that they were collected from on a local scale (see Table 6.4 and Fig. 6.12).

6.5. Discussion

In this study, the extent of the genetic variability in an approximately 300 bp region of Domain I and the first part of Domain II of the mt 16S rRNA gene was examined in *I. scapularis* collected from several localities in the northern U.S.A. and southern Canada. The potential utility of this molecular marker for assessing the phylogeography of the blacklegged tick was also investigated. To my knowledge, this is the first time that this genetic marker has been used in a study of the phylogeography of a tick species. These data were concatenated with those obtained in Chapters 2 and 3 of this thesis, as well as those of Krakowetz et al. (2011), and the resulting information was used to further examine the phylogeography of *I. scapularis*.

6.5.1. Genetic Diversity

6.5.1.1. The mt 16S rRNA gene (Domains I and II)

One hundred and thirty haplotypes of Domains I and II (i.e., the 5' end) of the mt 16S rRNA gene were detected among the 577 ticks collected from southern Canada, and the Midwest (i.e., Minnesota) and Northeast (i.e., Rhode Island) of the U.S.A. None of these haplotypes has been reported previously in other studies of *I. scapularis*. The number of haplotypes detected in

the present study was precisely two and a half times that detected for Domains IV and V (i.e., the 3' end) of the mt 16S rRNA gene in the study described in Chapter 2. This was despite 1) the same ticks being examined, except for five individuals in the current study due to failed sequencing reactions of the PCR-amplicons, and 2) the gene region in the present study being more than 100 bp shorter than that investigated in Chapter 2 (i.e., ~300 bp, as compared to ~400 bp, respectively). The number of haplotypes detected in this study was also substantially greater than that detected in several other studies of *I. scapularis* (i.e., 7-29 haplotypes) using 253-433 bp of the 3' end of the mt 16S rRNA gene as a genetic marker and samples sizes of 29-451 ticks (Rich et al. 1995, Norris et al. 1996, Qiu et al. 2002, Trout et al. 2009, Humphrey et al. 2010, Krakowetz et al. 2011, Van Zee et al. 2013).

The number of haplotypes detected in the present study based on analysis of sequence data of the 5' end of the mt 16S rRNA gene of *I. scapularis* was also considerably greater than that reported in other studies of *I. scapularis* in which the genetic variability in other mitochondrial and nuclear markers was examined (Norris et al. 1996, Mechai et al. 2013, Van Zee et al. 2013; Chapter 3). For example, nearly two and a half times the number of haplotypes was detected for *I. scapularis* in the present study as compared to that in a recent study by Mechai et al. (2013) in which the extent of the genetic variability in the mt *cox1* gene was examined for 414 blacklegged ticks. Furthermore, in a recent study by Van Zee et al. (2013), the occurrence of single-nucleotide polymorphisms (SNP) in nine nuclear genes, as well as the 3' end of the mt 16S rRNA gene, was assessed for 40 *I. scapularis* collected from Virginia, New Jersey, Georgia, and Mississippi in the U.S.A. It was determined that two of the nine nuclear markers (i.e., ixolaris 2A and defensin) were as highly variable as the mt 16S rRNA gene marker, and that the remaining seven nuclear markers were less variable, some substantially so (e.g., cytochrome c oxidase polypeptide) (Van Zee et al. 2013). Thus, it may be inferred that the 5' end of the mt 16S rRNA gene of *I. scapularis* is at least two and a half times as variable as any of the nine nuclear markers examined in the study by Van Zee et al. (2013). This hypothesis, however, requires extensive testing in additional studies of *I. scapularis* using larger samples sizes. Also, because a subset (i.e., 225) of the 577 ticks characterized using the 5' end of the mt 16S rRNA gene in this study was also examined using the mt 12S rRNA + $tRNA^{Val}$ genes in the study described in Chapter 3, it was possible to determine that the 5' end of the mt 16S rRNA gene was more variable than the mt 12S rRNA + $tRNA^{Val}$ genes for these ticks. This was

because the 61 haplotypes of the mt 12S rRNA + $tRNA^{Val}$ genes corresponded to 75 haplotypes of the 5' end of the mt 16S rRNA gene (i.e., that there were 23.0% more haplotypes detected in the same ticks based on sequences of the 5' end of the mt 16S rRNA gene as compared to the mt 12S rRNA + $tRNA^{Val}$ genes). Moreover, the fifty-seven ticks that represented the most commonly detected haplotype (i.e., haplotype CT08) based on sequences of the mt 12S rRNA + $tRNA^{Val}$ genes in Chapter 3 corresponded to 16 unique haplotypes based on sequences of the 5' end of the mt 16S rRNA gene (this study).

A large proportion (i.e., 65.4%) of the 130 haplotypes detected in the present study was represented by one or two ticks each. This suggests that there may be a large number of undetected haplotypes within the sampled areas. The rarefaction, extrapolation, and estimated total haplotype richness (Chao 2) curves support this prediction. The large proportion of singletons in the present study (i.e., 46.2% of the 130 total haplotypes; $n = 577$) differed from that for the 3' end of the mt 16S rRNA gene in Chapter 2 (i.e., 28.8% of the 52 total haplotypes; $n = 582$), but appeared to be similar to that for the mt 12S + $tRNA^{Val}$ genes in Chapter 3 (i.e., 48.4% of the 62 total haplotypes; $n = 229$), despite the examination of 60.3% fewer ticks in the latter study. However, the rarefaction, extrapolation, and estimated total haplotype richness (Chao 2) curves for the 5' end of the mt 16S rRNA gene suggest that the extent of the genetic variability in this genetic marker is greater than that for the mt 12S rRNA + $tRNA^{Val}$ genes (Chapter 3).

The number of nucleotide differences among the 130 haplotypes ranged from 1-17 bp when aligned over 286 bp, and most haplotypes differed from one another by 1 or 2 bp. These differences were greater than that reported in Chapters 2 (i.e., 1-5 bp; 408 bp alignment; 52 haplotypes; 582 ticks) and 3 (i.e., 1-13 bp; 430 bp alignment; 62 haplotypes; 229 ticks) for the mt 16S and 12S rRNA + $tRNA^{Val}$ genes, respectively. In the present study, the number of variable nucleotide positions in the alignment was 74, as compared to 42 and 41 variable positions in Chapters 2 and 3, respectively. This difference is probably due to the greater number of indels, or proportion of indels relative to variable positions, detected in the present study, as compared to those in Chapters 2 and 3. For example, fourteen indels comprising 19% of the 74 variable positions within the 286 bp alignment of the 5' end of the mt 16S rRNA gene were detected in the current study, whereas only four indels representing 10% of the 42 variable positions in the 408 bp alignment were detected for the 3' end of the gene in Chapter 2 and three indels

comprising 14% of the 41 variable positions in the 430 bp alignment were detected for the mt 12S rRNA + $tRNA^{Val}$ genes in Chapter 3.

In the present study, eight haplotypes (i.e., Hap_1, Hap_2, Hap_7, Hap_26, Hap_34, Hap_45, Hap_52, and Hap_72) comprised approximately half (i.e., 47%) of the ticks collected. Of these, the most common haplotype, Hap_2, represented 23% of the *I. scapularis*. In contrast, the most frequently detected haplotypes in Chapters 2 and 3, respectively, were Is-1, which comprised 49% of the ticks (n = 582), and CT08, which comprised 25% of the *I. scapularis* (n = 229). Haplotype Hap_2 also represented the central haplotype of the minimum spanning network, as did haplotypes Is-1 and CT08 in their respective networks in Chapters 2 and 3. The overall star-shaped pattern of the minimum spanning network in the present study is representative of rapidly expanding populations (Avise 2000, Qiu et al. 2002, Humphrey et al. 2010), which is consistent with the relatively recent establishment of populations of *I. scapularis* in the Midwestern U.S.A. (e.g., Godsey et al. 1987, Drew et al. 1988, French 1995, Riehle and Paskewitz 1996, Dennis et al. 1998, Sanders and Guilfoile 2000, Cortinas and Kitron 2006, Lee et al. 2013) and southern Canada (e.g., Watson and Anderson 1976, Barker and Lindsay 2000, Ogden et al. 2008c, Ogden et al. 2009, Bouchard et al. 2011). The pattern of the minimum spanning network in the present study is also consistent with the patterns of the haplotype networks corresponding to the 3' end of the mt 16S rRNA gene and 12S rRNA + $tRNA^{Val}$ genes in Chapters 2 and 3, respectively, as well as with that for the 3' end of the mt 16S rRNA gene of *I. scapularis* collected from the Northeastern and Midwestern regions of the U.S.A. in the relatively recent studies of Qiu et al. (2002) and Humphrey et al. (2010). Furthermore, a recent investigation of the population genetic structure of *I. scapularis* using the mt *cox1* gene as a molecular marker revealed a similarly shaped minimum spanning network (Mechai et al. 2013).

The ratio of transitional to transversional mutations for the 5' end of the mt 16S rRNA gene was approximately 1:1, which was consistent with that reported by Norris et al. (1996) for each of the mt 12S and 16S rRNA genes and with that determined in Chapter 3 for the mt 12S + $tRNA^{Val}$ genes, but different from the 2:1 ratio determined for the 3' end of the mt 16S rRNA gene in Chapter 2. A possible explanation, which was proposed in Chapter 2, for the 2:1 transition to transversion ratio observed for the 3' end of the mt 16S rRNA gene in that study, as compared to the 1:1 ratio of Norris et al. (1996), was that only *I. scapularis* belonging to the "American" clade were examined in the study reported in Chapter 2, whereas ticks

corresponding to both the "southern" and "American" clades were investigated in the study by Norris et al. (1996). In contrast, for the data reported in this thesis, the difference between the ratios of transitional to transversional mutational changes for the different regions (i.e., 1:1 for both the 5' end of the mt 16S rRNA and the mt 12S + $tRNA^{Val}$ genes, but 2:1 for the 3' end of the mt 16S rRNA gene) cannot be attributed to different clades or even to different ticks. This is because the 5' and 3' ends of the mt 16S rRNA genes were examined using the same ticks and the mt 12S + $tRNA^{Val}$ genes were investigated using a subset of these ticks. Instead, these discrepancies may be attributable to differences in the secondary structures of the rRNAs (plus tRNA in Chapter 3). It was determined in the study described in Chapter 2 that a region of the mt 16S rRNA gene of *I. scapularis* and of *I. ricinus* is hypervariable. Thus, there may be fewer structural constraints on the "hypervariable region," as compared to its neighbouring regions, possibly affecting the function of the ribosome (Misof et al. 2002). Furthermore, within the "hypervariable region" was a relatively equal number of transitions relative to transversions (Chapter 2). In contrast, the ratio of transitions to transversions was high at 11:2 within the flanking regions (Chapter 2). Thus, there appears to be an inverse relationship between the ratio of transitions to transversions and the level of genetic variability within these gene regions. This is reasonable, because in parts of the rRNAs (plus tRNA in Chapter 3) that are structurally constrained (i.e., tolerate fewer mutational changes), transitions are more conducive than transversions for the maintenance of base-pairing. In the present study, analysis of the mutational changes in relation to their position in the predicted secondary structure of the 5' end of the mt 16S rRNA gene was not conducted, although the secondary structure of the entire mt 16S rRNA gene was predicted for six *I. scapularis* in Chapter 5. The results in Chapter 5 suggest a relatively homogeneous distribution of the number and types (e.g., transitional and transversional) of mutational changes in Domains I and II of the mt 16S rRNA gene. Therefore, the difference between the ratios of transitional to transversional mutational changes in the different regions examined in this thesis probably reflects the tolerance of the different parts of the rRNAs (plus tRNA in Chapter 3) to mutational changes.

6.5.1.2. Concatenated datasets

A total of 179 haplotypes based on the concatenated sequence data (695 bp total) of Domains I and II (this study) and IV and V (studies described in Chapter 2 and by Krakowetz et

al. 2011) of the mt 16S rRNA gene was detected in 576 adult and nymphal *I. scapularis* collected from several localities in the Midwestern and Northeastern U.S.A. and southern Canada. Seven haplotypes (i.e., Is-1 + Hap_2, Is-15 + Hap_45, Is-1 + Hap_34 + Is-7 + Hap_52, Is-63 + Hap_45, Is-1 + Hap_26, and Is-13 + Hap_2) comprised 32% of the ticks. Of these, haplotype Is-1 + Hap_2 was the most common, representing 16% of the *I. scapularis*. This was expected, as haplotype Is-1 was the most frequently detected haplotype based on DNA sequences of Domains IV and V of the mt 16S rRNA gene in studies of *I. scapularis* conducted in southern Canada (Krakowetz et al. 2011), and the Midwest and Northeast of the U.S.A., as well as in the study described in Chapter 2. Also, haplotype CT08 was the most common haplotype based on DNA sequences of the mt 12S + $tRNA^{Val}$ genes in the study detailed in Chapter 3. Haplotype Is-1 + Hap_2 also represented the central haplotype in the minimum spanning network of the concatenated dataset in the present study. The overall star-shaped pattern of this network was consistent with that depicted for the 3' end of the mt 16S rRNA gene of northern (i.e., those of the "American" clade) *I. scapularis* (Qiu et al. 2002, Humphrey et al. 2010, Krakowetz et al. 2011; Chapter 2). The same minimum spanning network pattern was reported in recent studies based on the DNA sequences of the mt 12S + $tRNA^{Val}$ genes (Chapter 3) and the mt *cox1* gene (Mechai et al. 2013), suggesting that populations of *I. scapularis* in the Midwestern and Northeastern U.S.A. and southern Canada have undergone recent population expansions.

Analyses were also conducted on the combined sequence data of the mt 12S rRNA + $tRNA^{Val}$ genes (Chapter 3), as well as Domains I and II (this chapter), and IV and V of the mt 16S rRNA gene (Chapter 2 and Krakowetz et al. 2011) for 225 adult and nymphal *I. scapularis*. These revealed a total of 117 haplotypes, which were comprised of different combinations of 53 haplotypes of Domains IV and V of the mt 16S rRNA gene (Chapter 2 and Krakowetz et al. 2011), 61 haplotypes of the mt 12S rRNA + $tRNA^{Val}$ genes (Chapter 3), and 75 haplotypes of Domains I and II of the mt 16S rRNA gene (this chapter). These results suggest that the 5' end of the mt 16S rRNA gene (~300 bp) is more variable than its 3' end and the mt 12S rRNA + $tRNA^{Val}$ genes, as well as that 430 bp of the mt 12S rRNA + $tRNA^{Val}$ genes is more variable than the 3' end of the mt 16S rRNA gene (~400 bp) for these 225 ticks.

Contrary to the results for the other gene regions (including the concatenated datasets) of *I. scapularis* examined in this thesis, the most common haplotype of the three concatenated gene regions (i.e., Is-15 + CT07 + Hap_45), comprising 9% of the ticks in the present study, did not

represent the central haplotype in the minimum spanning network. Furthermore, the number of detected haplotypes that differed directly by 1-3 bp from the most common haplotype in the dataset was two, as compared to 20 haplotypes that differed directly from the second most common haplotype, Is-1 + CT08 + Hap_2, which represented the central haplotype in the network. Since more than twice as many ticks from Long Point Provincial Park (ON) were included in this dataset versus any of the other localities, and haplotype Is-15 + CT07 + Hap_45 comprised nearly one third of the haplotypes detected there, the overall frequency of this haplotype was high. Consequently, the overall frequency of haplotype Is-15 + CT07 + Hap_45 surpassed the frequency of haplotype Is-1 + CT08 + Hap_2 across the localities, despite the increased resolution of the ticks using the three DNA fragments. Haplotype Is-15 + CT07 + Hap_45 was also detected in Point Pelee National Park (ON), although in low frequency, and was absent from the other localities. The high frequency of this haplotype in Long Point Provincial Park (ON) may be due in part to sampling bias and/or the dispersal patterns of the ticks via migratory birds.

6.5.2. Phylogeography

The phylogeographical structures of several tick species, including *I. scapularis*, have been examined using both mtDNA (e.g., 16S rRNA, 12S rRNA, *cox1*, *cox2*, *cox3*, *cytb*, and the control region) and nDNA (e.g., *EF1-α*, defensin, and *TROSPA*; microsatellites; and the ITS2) in recent studies (e.g., Qiu et al. 2002, Rosenthal and Spielman 2004, Casati et al. 2008, Kempf et al. 2009, Trout et al. 2009, Humphrey et al. 2010, Kempf et al. 2011, Krakowetz et al. 2011, Noureddine et al. 2011, Song et al. 2011, Cangi et al. 2013, Mechai et al. 2013, Dinnis et al. 2014). However, mtDNA is most commonly used for such investigations (e.g., Qiu et al. 2002, Casati et al. 2008, Kempf et al. 2009, Trout et al. 2009, Humphrey et al. 2010, Krakowetz et al. 2011, Noureddine et al. 2011, Song et al. 2011, Cangi et al. 2013, Mechai et al. 2013, Dinnis et al. 2014). The study in Chapter 2 showed that the 3' end of the mt 16S rRNA gene offers partial resolution of the spatial distributional patterns of *I. scapularis*, particularly with respect to the eastern (i.e., including the provinces of Ontario, Quebec, New Brunswick, Nova Scotia, Prince Edward Island, and Newfoundland in Canada, as well as the state of Rhode Island in the U.S.A.) and western (i.e., including the provinces of Alberta, Saskatchewan, and Manitoba in Canada, as well as the state of Minnesota in the U.S.A.) geographical regions. However, a large proportion

(i.e., 49%) of the ticks (*n* = 582) collected from eleven different localities represented a single haplotype (i.e., Is-1), which was detected in nine of these localities and both the eastern and western geographical regions. Several other haplotypes were also shared between the eastern and western geographical areas. In the study in Chapter 3, it was demonstrated that the mt 12S rRNA + $tRNA^{Val}$ genes offer greater resolution of the spatial distribution of *I. scapularis* across Canada and in Minnesota (i.e., Midwestern) and Rhode Island (i.e., Northeastern) in the U.S.A. Again, however, a large proportion (i.e., 25%) of the ticks (*n* = 229) comprised a single haplotype (i.e., CT08), which was detected in four of the eight localities and both the eastern and western geographical areas in that study. Furthermore, seven additional haplotypes were shared between the eastern and western geographical regions. Concatenation of the DNA sequences of the 3' end of the mt 16S rRNA gene (Is series) with that of the mt 12S rRNA + $tRNA^{Val}$ genes (CT series) in the study described in Chapter 3, showed that those ticks that had been previously characterized as Is-1 in Chapter 2 (*n* = 82) comprised 31 haplotypes of the mt 12S rRNA + $tRNA^{Val}$ genes, most of which could now be associated with the eastern or western geographical areas only. Not surprisingly, however, 40% of these ticks (14% of the ticks overall) represented a single haplotype (i.e., haplotype Is-1 + CT08), which was also detected in four of the eight localities and both the eastern and western geographical regions. Only two additional haplotypes of the concatenated dataset were also shared between the eastern and western geographical regions in that study. In the study described in Chapter 5, it was determined that the 5' end of the mt 16S rRNA gene may represent a useful genetic marker for examining the geographical origins in the U.S.A. of *I. scapularis* in southern Canada, in addition to the most commonly used gene region (i.e., ~400 bp of Domains IV and V). More importantly, though, it was determined that two ticks of the same frequently detected haplotype (i.e., Is-1) based on sequences of the 3' end of the mt 16S rRNA gene, which was present in both the eastern and western geographical regions, could be distinguished from one another based on their respective sequences of Domains I and II of the gene. Therefore, the aims of this study were twofold: 1) to determine the extent of the genetic variation in the 5' end of the mt 16S rRNA gene of blacklegged ticks and whether this region, alone, may be useful for determining the geographical origins in the U.S.A. of blacklegged ticks in southern Canada, and 2) to determine whether the sequence data for the 5' end of the mt 16S rRNA gene may offer further resolution of the spatial dispersal patterns of

these ticks, especially with respect to those *I. scapularis* that have been previously characterized as haplotype Is-1 based on DNA sequences of the 3' end of the mt 16S rRNA gene.

In the present study, it was determined that the 5' end of the mt 16S rRNA gene offers some resolution of the dispersal patterns of blacklegged ticks in southern Canada from the northern U.S.A. However, a single haplotype (i.e., Hap_2), which was detected in six of eleven localities and both the eastern and western geographical regions, still comprised a relatively large proportion (i.e., 23%) of the 577 ticks examined using the 5' end of the mt 16S rRNA gene. Additionally, a small number of haplotypes (i.e., 13 haplotypes including haplotype Hap_2) representing 47% of the *I. scapularis* were shared between the eastern and western geographical regions. Concatenation of the 5' (Hap series) and 3' (Is series) ends of the mt 16S rRNA gene for 576 blacklegged ticks increased the resolution of the spatial patterns of these ticks, as the most common haplotype of this dataset (i.e., haplotype Is-1 + Hap_2) comprised only 16% of the ticks and was detected in five of the eleven localities and both the eastern and western geographical areas. For this dataset, the number of haplotypes that were detected in both the eastern and western geographical areas, which collectively represented 26% of the *I. scapularis*, was eight, including haplotype Is-1 + Hap_2. Even greater resolution was achieved when all three gene regions (i.e., the 5' and 3' ends of the mt 16S rRNA gene with the mt 12S rRNA + $tRNA^{Val}$ genes) were concatenated. In fact, the resolution of the ticks via this dataset was so great that a haplotype found only in Ontario (i.e., Is-15 + CT07 + Hap_45) was the most common haplotype overall, representing 9% of the 225 ticks characterized, whereas haplotype Is-1 + CT08 + Hap_2, which was the only haplotype to be detected in both the eastern and western geographical regions, represented 8% of the ticks. As discussed in the genetic diversity section above, the high overall frequency of haplotype Is-15 + CT07 + Hap_45 is probably a consequence of the high frequency of this haplotype in Long Point Provincial Park (ON) and/or the large number of ticks from this locality relative to the others in this dataset. Sampling bias and/or the dispersal patterns of the ticks via migratory birds may also have affected these observations. Thus, the power of resolution to identify geographical patterns of spread of *I. scapularis* has generally increased with each subsequent molecular marker investigated during my PhD studies (i.e., the nuclear marker examined in Chapter 4 was the exception to this trend) and with the level of data concatenation (i.e., with respect to whether two or three gene regions were utilized).

Previously, other research groups have attempted to obtain greater phylogeographical signal for tick species of medical and veterinary importance (e.g., *I. scapularis* and *I. ricinus*) in order to better understand their spatial distributions by concatenating their datasets (e.g., Norris et al. 1996, Noureddine et al. 2011, Dinnis et al. 2014). However, the phylogenetic and phylogeographical outcomes of the concatenated datasets were often identical or nearly identical to the outcomes of those associated with any one of the constituent DNA sequence fragments (e.g., Norris et al. 1996, Noureddine et al. 2011). These results are in agreement with those of the present study, because the minimum spanning networks for the one-, two-, and three-genetic marker datasets were relatively similar. Each network featured a central haplotype from which many haplotypes (i.e., 20-28 haplotypes) directly differed by 1-3 bp. The overall star-shaped patterns of these networks is consistent with the expected configuration for an abundant species that has recently expanded its distributional range from a small number of founders (Avise 2000). Numerous phylogroups corresponding to "eastern" haplotypes or those associated with both the "eastern" and "western" geographical regions were also identified among the haplotypes of each minimum spanning network. The relative similarity of these groupings across the networks may be evidence of spatial clusters of ticks that arose, in part, as a consequence of genetic bottlenecks resulting from founder events, as well as single mutational changes in the DNA sequences of the mitochondrial genes. The starburst patterns observed in the networks also represent evidence of several different founder events and numerous single mutational changes. Furthermore, each minimum spanning network revealed a shallow genealogical structure for *I. scapularis* (Avise 2000).

The results of the present study are consistent with the life history of *I. scapularis* in northeastern North America in terms of its dispersal patterns as facilitated by migratory passerines (e.g., Weisbrod and Johnson 1989, Scott et al. 2001, Marra et al. 2005, Ogden et al. 2008c, Taylor et al. 2011, Scott et al. 2012, Stanley et al. 2012, Kirsch et al. 2013). For example, it has been determined by examining the behaviour of migratory birds while at a stopover site (i.e., a site where birds replenish themselves for subsequent flights) at Long Point, ON that migratory bird individuals tend to relocate themselves within the same landscape during their stopovers, covering distances as great as 30 km from where they were initially captured (Taylor et al. 2011). This behaviour by some species of migratory bird (e.g., Swainson's Thrush, *Catharus ustulatus*; and, Hermit Thrush, *Catharus guttatus*), which have been captured at bird

observatories in eastern Canada including Pelee Island and Long Point in Ontario and found to be infested with immature *I. scapularis*, may, in part, explain the high prevalence of haplotype Is-15 + CT07 + Hap_45 in Long Point Provincial Park, the low prevalence of this haplotype in nearby Point Pelee National Park, and the absence of this haplotype in the other localities (Ogden et al. 2008c, Taylor et al. 2011). The behaviour of migratory birds may also account for the differences in the genetic structure of *I. scapularis* between populations in the "western" (i.e., Manitoba, Canada and Minnesota, U.S.A.) and "eastern" (eastern Canada and Rhode Island, U.S.A.) geographical areas, as determined using a small number of mitochondrial markers (i.e., *cox1*, 16S rRNA, and 12S rRNA + $tRNA^{Val}$) (Krakowetz et al. 2011, Mechai et al. 2013; Chapter 2; Chapter 3). For example, numerous long-distance migratory bird species have been captured frequently (i.e., ≥ 10 individuals per year) over a period of 40 years (i.e., minimum of 10 years) at the Powdermill Nature Reserve in Pennsylvania (U.S.A.) and/or the Long Point Bird Observatory in Ontario (Marra et al. 2005). The majority of these bird species were captured at both observatories, suggesting that birds that pass through Pennsylvania probably also pass through southeastern Ontario during their spring migration (Marra et al. 2005). However, the tracking of individual migratory songbirds (i.e., Wood Thrush, *Hylocichla mustelina*) has revealed that the migration route, as compared to migration timing, yields relatively low repeatability in spring and autumn (Stanley et al. 2012). Thus, the migration route of individual birds may be influenced by several biotic (e.g., its physical condition) and abiotic (e.g., local conditions, such as weather patterns) factors (Stanley et al. 2012).

In the present study, differences in the genetic structure between ticks collected from the "eastern" and "western" geographical regions were also apparent. For example, 65 (83%) of the 78 haplotypes of the 5' end of the mt 16S rRNA gene found in the "western" region (i.e., Prairie Provinces and Minnesota) were not found in the "eastern" region (Central and Atlantic Provinces, and Rhode Island). Similarly, most (i.e., 80%) of the 65 haplotypes of the 5' end of the mt 16S rRNA gene in the "eastern" area were not found in the Prairie Provinces or Minnesota. Furthermore, only six haplotypes (i.e., 5%) of the 5' end of the mt 16S rRNA gene were shared between the two geographical areas. Comparisons of the haplotypes of the 5' end of the mt 16S rRNA gene that were detected in each of four geographical regions (i.e., "western" Canada, Minnesota, "eastern" Canada, and Rhode Island) revealed that a greater proportion of haplotypes (i.e., approximately twice as many haplotypes) were shared between "western"

Canada and Minnesota and "eastern" Canada and Rhode Island than between "western" and "eastern" Canada or Minnesota and Rhode Island. For example, shared haplotypes between "western" Canada and Minnesota comprised 56% of the 43 haplotypes detected in "western" Canada and 41% of the 59 haplotypes detected in Minnesota, whereas shared haplotypes between "western" and "eastern" Canada comprised 26% of the 43 haplotypes detected in "western" Canada and 22% of the 49 haplotypes detected in "eastern" Canada. Shared haplotypes between Minnesota and Rhode Island comprised 14% of the 59 haplotypes found in Minnesota and 25% of the 32 haplotypes detected in Rhode Island. The proportion of shared haplotypes between "eastern" Canada and Rhode Island was 33% for "eastern" Canada and 50% for Rhode Island. These results support the hypothesis that *I. scapularis* introduced into the Prairie Provinces of Canada (i.e., Alberta, Saskatchewan, and Manitoba) may have originated from established populations in the Upper Midwest (i.e., Minnesota and Wisconsin) of the U.S.A., whereas those introduced into the Central and Atlantic Provinces of Canada (i.e., Ontario and Quebec, and New Brunswick, Prince Edward Island, Nova Scotia, and Newfoundland, respectively) may have originated from endemic populations in the Northeast of the U.S.A. in relation to the different routes (i.e., flyways) taken by passerines during their spring migrations (Krakowetz et al. 2011, Scott et al. 2012). This hypothesis is also supported by the concatenated dataset for the mt 12S rRNA + $tRNA^{Val}$ genes (Chapter 3), as well as Domains I and II (this chapter), and IV and V of the mt 16S rRNA gene (Chapter 2 and Krakowetz et al. 2011) of *I.* scapularis in the present study, as there was geographical variation in the number of haplotypes that were detected in "western," as compared to "eastern" populations of *I. scapularis*. For example, for those populations where sample sizes exceeded 30 ticks (i.e., Camp Ripley, St. Croix State Park, and Long Point Provincial Park), the number of haplotypes per population detected in Camp Ripley (26 haplotypes; $n = 36$) and St. Croix State Park (20 haplotypes; $n = 31$), Minnesota was approximately twice as great as that in Long Point Provincial Park (25 haplotypes; $n = 66$). There were also several shared haplotypes of the mt 12S rRNA + $tRNA^{Val}$ genes + 5' and 3' ends of the mt 16S rRNA gene among the nine established populations and among the eight Canadian provinces and states in the U.S.A., but only one (i.e., haplotype Is-1 + CT08 + Hap_2) that was shared between the "western" and "eastern" geographical areas, which further support the flyway hypothesis. Nonetheless, the pie charts representing the haplotypes of ticks from the nine established populations suggest that there is limited gene flow among them.

The presence of shared haplotypes between the "western" and "eastern" geographical areas may be explained by the usage of multiple flyways (e.g., Mississippi and Atlantic flyways) by migratory bird species that are parasitized by immature (i.e., larval and nymphal) *I. scapularis*. For example, the Common Yellowthroat (*Geothlypis trichas*), which is known to be infested with larval and nymphal *I. scapularis* (Weisbrod and Johnson 1989, Scott et al. 2001, Scharf 2004, Ogden et al. 2008c), has been captured in southern Manitoba, Canada (Scott et al. 2001) and Minnesota, U.S.A. (Weisbrod and Johnson 1989) along the Mississippi flyway (Scott et al. 2012), but also in south-Central Canada (Scott et al. 2001, Marra et al. 2005, Ogden et al. 2008c), Pennsylvania, U.S.A. (Marra et al. 2005), Maryland, U.S.A. (Scharf 2004), and Nova Scotia, Canada (Scott et al. 2001) along the Atlantic flyway (Scott et al. 2012). Many other species of migratory bird that are also known to be infested with *I. scapularis* (Weisbrod and Johnson 1989, Scott et al. 2001, Scharf 2004, Ogden et al. 2008c, Scott et al. 2012), such as the Yellow Warbler (*Dendroica petechia*), the Magnolia Warbler (*Dendroica magnolia*), the Nashville Warbler (*Vermivora ruficapilla*), the Canada Warbler (*Wilsonia canadensis*), the Mourning Warbler (*Oporornis philadelphia*), the Wilson's Warbler (*Wilsonia pusilla*), the Yellow-rumped Warbler (*Dendroica coronate*), the American Redstart (*Setophaga ruticilla*), the Swainson's Thrush (*Catharus ustulatus*), the Northern Waterthrush (*Seiurus noveboracensis*), the Gray Catbird (*Dumetella carolinensis*), the Veery (*Catharus fuscensens*), the Ovenbird (*Seiurus aurocapillus*),the House Wren (*Troglodytes aedon*), the White-throated Sparrow (*Zonotichia albicollis*), and the Swamp Sparrow (*Melospiza geogiana*) have also been captured in Canadian provinces and/or states in the U.S.A. that correspond to both the Mississippi and Atlantic flyways (Weisbrod and Johnson 1989, Scott et al. 2001, Scharf 2004, Marra et al. 2005, Ogden et al. 2008c, Scott et al. 2012, Suomala et al. 2012).

The differences in the genetic structure between *I. scapularis* collected from the "eastern" and "western" geographical regions may, in contrast, be explained by the primary usage of a single flyway (i.e., Mississippi or Atlantic flyway) by tick-infested migratory bird species. For example, numerous species of migratory bird that are known to be parasitized by immature *I. scapularis* have been captured in Central and Atlantic Canada, Pennsylvania, Maryland, and/or Maine, but not in the Prairie Provinces or Minnesota (Scott et al. 2001, Scharf 2004, Marra et al. 2005, Ogden et al. 2008c, Scott et al. 2012, Suomala et al. 2012). Although this data is not comprehensive and, therefore, not conclusive, bird species such as the Wood Thrush (*Hylocichla*

mustelina), the Black-throated Blue Warbler (*Dendroica caerulescens*), the Chestnut-sided Warbler (*Dendroica pensylvanica*), the Blue-winged Warbler (*Vermivora pinus*), the Hooded Warbler (*Wilsonia citrina*), the Indigo Bunting (*Passerina cyanea*), the White-throated Sparrow (*Zonotrichia albicollis*), the Kentucky Warbler (*Oporornis formosus*), the Red-Winged Blackbird (*Aegelaius phoniceus*), the Song Sparrow (*Melospiza melodia*), the Brown Thrasher (*Toxostoma rufum*), the Field Sparrow (*Spizella pusilla*), the Eastern Towhee (*Pipilo erythropthalmus*), the Golden-crowned Kinglet (*Regulus satrapa*), the Ruby-crowned Kinglet (*Regulus calendula*), the Cedar Waxwing (*Bombycilla cedrorum*), and the Lincoln's Sparrow (*Melospiza lincolnii*), which are known to be parasitized by larval and nymphal *I. scapularis* (Scott et al. 2001, Scharf 2004, Ogden et al. 2008c, Scott et al. 2012), appear to primarily use the Atlantic flyway (Scott et al. 2001, Scharf 2004, Marra et al. 2005, Ogden et al. 2008c, Scott et al. 2012, Suomala et al. 2012). Since an estimated 50 to 175 million blacklegged ticks are being transported into Canada by migratory birds each year (Ogden et al. 2008b), at the present time, the flyway hypothesis represents the most reasonable explanation for the phylogeographical patterns that have observed in the present study, as well as in several others (Krakowetz et al. 2011, Mechai et al. 2013; Chapter 2; Chapter 3).

6.5.3. Conclusions

The genetic diversity within a newly characterized portion (~280 bp) of the mt 16S rRNA gene (i.e., Domains I and II) among individuals of *I. scapularis* collected from several localities in southern Canada and the Midwest and Northeast of the U.S.A. was substantially greater than that detected in Domains IV and V (~410 bp) of the mt 16S rRNA gene (Chapter 2) and in the mt 12S rRNA + $tRNA^{Val}$ genes (~430 bp) (Chapter 3). It also appears that the extent of the genetic variability within the 5' end of the mt 16S rRNA gene of *I. scapularis* is greater than that in a 500-657 bp region of the mt *cox1* gene of blacklegged ticks (Mechai et al. 2013), as well as several nuclear genes of *I. scapularis* analyzed in a recent study by Van Zee et al. (2013). There may also be a large number of undetected haplotypes, as determined using the Chao 2 estimator, within the sampled areas. The 5' end of the mt 16S rRNA gene offered some resolution of the dispersal patterns of blacklegged ticks in southern Canada; however, a single haplotype (i.e., Hap_2) represented 23% of the ticks examined and was present in both the eastern and western geographical areas. Thus, although Domains I and II of the mt 16S rRNA gene may only provide

limited phylogeographical signal for *I. scapularis*, this marker may be very useful for population genetic studies of blacklegged ticks.

Concatenation of Domains I and II with Domains IV and V offered greater resolution of the phylogeographical patterns among the *I. scapularis*, in part, because the most common haplotype, which occurred in both the eastern and western geographical regions, comprised only 16% of the ticks examined. Furthermore, concatenation of these two gene regions with that of the mt 12S rRNA + $tRNA^{Val}$ genes revealed additional resolution of the phylogeographical relationships among the blacklegged ticks, also, in part, because the most common haplotype that was present in both the eastern and western geographical regions comprised a mere 8% of the ticks studied.

The patterns of the minimum spanning networks for the one-, two-, and three-marker datasets were similar, which may be evidence of spatial clusters of ticks that arose, as a consequence of genetic bottlenecks resulting from multiple founder events, as well as multiple single mutational changes in the DNA sequences of the mitochondrial genes of these ticks. The pattern of these networks was also indicative of a shallow genealogical structure and recent population expansion for *I. scapularis*, which is consistent with the life history of blacklegged ticks in the northern U.S.A. and southern Canada. There was a clear difference in the genetic structure, based on the three markers examined in the present study, but especially the 1126 bp marker, between ticks collected from the "eastern" and "western" geographical areas. This difference supports the hypothesis that blacklegged ticks introduced into the Prairie Provinces of Canada may have originated from resident populations in the Upper Midwest of the U.S.A., while those *I. scapularis* establishing within the Central and Atlantic Provinces of Canada may have originated from resident populations in the Northeast of the U.S.A., as a result of the different flyways taken by migratory passerines during their spring migrations. Therefore, although there were several shared haplotypes among the populations of *I. scapularis* examined, there was also limited gene flow among them, suggesting that the presence of these shared haplotypes is the result of migratory birds utilizing multiple flyways. Thus, the markers examined in the present study offer the much-needed resolution necessary to understand the population genetics of and phylogeographical relationships among *I. scapularis*, especially with respect to its trajectories of spread, and warrant further investigation in subsequent studies of this arthropod species of medical and veterinary importance.

In the next chapter, I will further investigate the geographical differences among tick populations by studying the phylogeography of *A*. *phagocytophilum*, an important human and animal pathogen vectored by *I*. *scapularis*.

CHAPTER 7
DEVELOPMENT OF PCR-BASED ASSAYS TO DISTINGUISH BETWEEN THE AP-HA AND AP-VARIANT 1 STRAINS OF *ANAPLASMA PHAGOCYTOPHILUM*, AND THE PREVALENCE OF THESE STRAINS IN BLACKLEGGED TICKS (*IXODES SCAPULARIS*) WITHIN CANADA[5]

7.1. Abstract

The prevalence of *Anaplasma phagocytophilum* in blacklegged ticks (*Ixodes scapularis*) from southern Canada was determined by PCR. PCR-based assays were also developed to distinguish a known human pathogenic strain of the bacterium (Ap-ha) from a strain (Ap-variant 1) that may not be associated with human infection. The proportion of ticks infected with *A. phagocytophilum* varied significantly among geographical regions and collection years, with a greater prevalence of infection in 2010 than in three preceding years. Furthermore, a greater proportion of infected ticks from Manitoba contained the Ap-ha strain than did infected ticks in more eastern provinces. These results suggest that human granulocytic anaplasmosis may represent an emerging disease in southern Canada and that the risk of human exposure to the Ap-ha strain may differ among geographical regions. The PCR-based assays developed in the present study provide valuable tools to monitor the prevalence of a human pathogenic strain of *A. phagocytophilum* in blacklegged ticks.

7.2. Introduction

The tick-borne bacterium *Anaplasma phagocytophilum*, previously known as *Ehrlichia equi* and *Ehrlichia phagocytophilum*, is the causative agent of human granulocytic anaplasmosis (HGA) in the U.S.A. (Chen et al. 1994). This non-specific febrile illness can sometimes be fatal (Hardalo et al. 1995, Dumler and Bakken 1998, Dumler et al. 2005, Bakken and Dumler 2006, Bakken and Dumler 2008, Dahlgren et al. 2011). The annual incidence rates of human infection

[5] Part of this chapter was reproduced with permission from the Centers for Disease Control and Prevention (http://wwwnc.cdc.gov/eid/page/copyright-and-disclaimers): **Krakowetz CN, Dibernardo A, Lindsay LR, Chilton NB (2014)** Two *Anaplasma phagocytophilum* strains in *Ixodes scapularis* ticks, Canada. Emerg Infect Dis 20: 2064-2067. NBC conceived the project. LRL organized the collection of samples. CNK and AD carried out laboratory work. All authors performed the data analyses, interpreted the data, wrote the manuscript, and approved the final manuscript.

with *A. phagocytophilum* in the U.S.A. has been on the increase in recent years (Dahlgren et al. 2011), with most (~90%) cases having been reported from Minnesota and Wisconsin in the Upper Midwest, and New York, Connecticut, Massachusetts, Rhode Island, and New Jersey in the Northeast (Dahlgren et al. 2011). Several strains of *A. phagocytophilum*, characterized based on sequences of the 16S rRNA gene, have been identified in ticks and mammals from different regions of the U.S.A. (Chen et al. 1994, Belongia et al. 1997, Massung et al. 1998, Massung et al. 2002, Courtney et al. 2003, Massung et al. 2003b, Massung et al. 2005, Poitout et al. 2005, Michalski et al. 2006, Steiner et al. 2008, Baldridge et al. 2009). In the Upper Midwest and Northeast, blacklegged ticks (*Ixodes scapularis*) are the vectors of a human pathogenic strain (Ap-ha) and a variant strain (Ap-variant 1) of *A. phagocytophilum* (Massung et al. 1998, Massung et al. 2002, Courtney et al. 2003, Michalski et al. 2006, Steiner et al. 2008), the latter of which appears not to be associated with human infection (Chen et al. 1994, Massung et al. 1998). Although only a partial sequence (375 bp) of the 16S rRNA gene has been determined for the Ap-variant 1 strain, compared to an almost complete sequence of this gene (1,433 bp) for the Ap-ha strain, the two strains differ in sequence from one another at two nucleotide positions near the 5' end of the 16S rRNA gene (Chen et al. 1994, Massung et al. 1998). There are also biological differences between the two strains, in that white-footed mice (*Peromyscus leucopus*) are the primary reservoirs of the Ap-ha strain (Telford et al. 1996, Levin et al. 2002), whereas white-tailed deer (*Odocoileus virginiansus*) are the primary reservoirs of the Ap-variant 1 strain (Massung et al. 2005). It has also been shown that there are marked differences in the prevalence of the Ap-ha and Ap-variant 1 strains in *I. scapularis* from different regions of eastern U.S.A., and within tick populations collected and tested in different years (Massung et al. 1998, Massung et al. 2002, Courtney et al. 2003, Massung et al. 2003b, Massung et al. 2005, Michalski et al. 2006, Steiner et al. 2008).

Several geographically isolated populations of *I. scapularis* have recently become established in southern Canada (Barker and Lindsay 2000, Ogden et al. 2008c, Ogden et al. 2008d, Ogden et al. 2009); however, there is little published information on the occurrence of *A. phagocytophilum* in these populations, except for a report of an infected *I. scapularis* on Long Point Peninsula, Ontario (ON) that had fed on a white-tailed deer (Drebot et al. 2001). Recently, an examination of *I. scapularis* on white-tailed deer in southwestern Quebec (Bouchard et al. 2013), an area where new populations of blacklegged tick are becoming established (Bouchard et

al. 2011, Koffi et al. 2012), revealed that 15% of ticks were infected with *A. phagocytophilum*, but strain types were not determined. A small proportion (<1%) of *I. scapularis* nymphs transported into Canada from the U.S.A. by migratory passerine birds are known to be infected with *A. phagocytophilum* (Ogden et al. 2008c); however, it has not been determined if one or both strains are carried by these ticks. Reliable estimates of the prevalence of the Ap-ha strain of *A. phagocytophilum* within different resident populations of *I. scapularis* across southern Canada and in adventitious ticks transported by migratory passerines from the U.S.A. into Canada is essential in order to assess the potential risk of exposure for Canadians to the pathogen that causes HGA. Currently, the approach most frequently used to distinguish *A. phagocytophilum* strains found in *I. scapularis* is to sequence the amplicons of the 16S rRNA gene produced by PCR analyses (Massung et al. 1998, Massung et al. 2002, Courtney et al. 2003, Massung et al. 2003b, Massung et al. 2005, Michalski et al. 2006, Steiner et al. 2008). Alternative PCR-based methods, such as restriction fragment length polymorphism (RFLP) analysis and single nucleotide polymorphism (SNP) genotyping, may provide less time-consuming and more cost-effective approaches to characterize the *A. phagocytophilum* strains within individual ticks.

Therefore, the objectives of the present study were to determine the proportion of blacklegged ticks infected with *A. phagocytophilum* in different geographical regions of Canada, and to develop restriction fragment length polymorphism (RFLP) and single nucleotide polymorphism (SNP) genotyping assays, targeting the 16S rRNA gene, to differentiate the Ap-ha strain from the Ap-variant 1 strain. We assessed the usefulness of these assays and determined the prevalence of the Ap-ha strain in *I. scapularis* from different geographical regions of Canada.

7.3. Materials and methods

7.3.1. Samples and DNA extraction

A total of 12,606 *I. scapularis* were collected as part of a national passive tick surveillance program conducted in Canada by the National Microbiology Laboratory (NML). Ticks were obtained from hosts or the environment in nine provinces from 2007 to 2010 (Table 7.1). Adult *I. scapularis* (n = 11) were also collected by drag sampling (Lindsay et al. 1999b) in 2009 from the Wainfleet Bog Conservation area and St. Lawrence Islands National Park in southern Ontario. Total genomic (g) DNA was extracted from each individual tick (at NML)

Table 7.1. The number of *A. phagocytophilum* PCR-positive blacklegged ticks collected in different Canadian provinces in different years.

Province	2007		2008		2009		2010		All 4 years combined		
	No. of ticks	No. PCR +ve	No. of ticks	No. PCR +ve	No. of ticks	No. PCR +ve	No. of ticks	No. PCR +ve	No. of ticks	No. PCR +ve	(%)
Western region											
Alberta	9	0	25	0	13	0	42	3	89	3	(3.4)
Saskatchewan	5	2	3	1	1	1	1	0	10	4	(40.0)
Manitoba	35	2	156	4	119	6	260	20	570	32	(5.6)
Central region											
Ontario	1,187	4	1,402	3	856	3	962	3	4,407	13	(0.3)
Quebec	982	13	1,687	26	1,026	8	1,002	24	4,697	71	(1.5)
Atlantic region											
New Brunswick	129	1	174	0	189	4	271	10	763	15	(2.0)
Nova Scotia	201	2	394	4	378	4	676	9	1,649	19	(1.6)
Prince Edward Island	54	3	76	1	107	3	122	3	359	10	(2.8)
Newfoundland	11	0	9	0	15	1	27	1	62	2	(3.2)
Total	2,613	27	3,926	39	2,704	30	3,363	73	12,606	169	(1.3)

using either the QIAamp DNA Mini Kit™ or the DNeasy Blood & Tissue Kit™ (Qiagen, Toronto, ON, Canada) as per the manufacturer's instructions, but with the modifications described previously (Ogden et al. 2006c). This extraction method was also used to isolate gDNA of *A. phagocytophilum* from an equine isolate (MN-93) that had been propagated in the HL-60 promyelocytic cell line (ATCC CCL-240) and used as a positive control in the PCR assays conducted at the NML.

At the University of Saskatchewan (U of S), total gDNA was extracted and purified, as previously described in Chapter 2, from each of the 168 questing *I. scapularis* adults collected in 2008 by drag sampling (Lindsay et al. 1999b) at three localities in Minnesota (U.S.A.); Itasca State Park (ISP), Camp Ripley (CR), and St. Croix State Park (CSP) (i.e., 28 female and 28 male ticks per locality). Total gDNA was also extracted and purified from 90 questing adult ticks collected by drag sampling in 2010 from two localities in southern Manitoba; within the Pembina Valley Provincial Park (PVPP) (i.e., 21 females and 25 males), and along the Stanley Trail (ST) (i.e., 19 females and 25 males), approximately 10 km south of the town of Morden.

7.3.2. Prevalence of *A. phagocytophilum*-infected *I. scapularis* in Canada

The gDNA of all 12,606 *I. scapularis* collected as part of the national passive surveillance program were screened by real-time PCR targeting the *msp2* gene (Courtney et al. 2004) to determine if they were infected with *A. phagocytophilum*. Samples that were PCR-positive for *A. phagocytophilum* using the *msp2* real-time PCR assay were confirmed using primers (Ap16Sf: 5'-GCTGCTTTTAATACTGCCAGA-3' and Ap16Sr: 5'-TCAGTACCGGAA CCAGATAGC-3') and probes (Ap 16S FAM-BBQ: 5'-CCACTGGTGTTCCTCCTAATATCT ACGA-3') in a real-time PCR targeting the 16S rRNA gene of *A. phagocytophilum* (Ogden et al. 2008c, Bouchard et al. 2013). Contingency tests (χ^2) were performed to determine if the proportions of ticks infected with *A. phagocytophilum* differed among collection years and/or the province in which they were collected.

7.3.3. Comparison of the 16S rRNA sequences of the Ap-ha and Ap-variant 1 strains

Although it has been shown previously that the Ap-variant 1 strain differs from the Ap-ha strain by 2 bp in part (375 bp) of the 16S rRNA gene (Chen et al. 1994, Massung et al. 1998), there is no information as to whether these two strains differ in sequence in other regions of the

16S rRNA gene, because only a small part of this gene has been characterized for the Ap-variant 1 strain. Thus, sequences of a larger part (870 bp) of the 16S rRNA gene were obtained for *A. phagocytophilum* present in some of the 168 *I. scapularis* adults collected from Minnesota. These samples were selected, because both (i.e., the Ap-variant 1 and Ap-ha) strains have been detected in *I. scapularis* from the Upper Midwest U.S.A. (Michalski et al. 2006).

The presence/absence of *A. phagocytophilum* in each of the 168 ticks was determined by targeting part of the bacterial 16S rRNA gene using nested PCR (nPCR). Primers EC12 (5'-TGA TCCTGGCTCAGAACGAACG-3') and EC9 (5'-TACCTTGTTACGACTT-3') (Kawahara et al. 2006) were used in the first phase, while primers HGE1F (5'-GGATTATTCTTTATAGCT TGCT-3') and HGE3R (5'-TTCCGTTAAGAAGGATCTAATCTC-3') (Adelson et al. 2004) were used in the second phase of the nPCR. All PCRs were conducted in 25 µl reaction volumes containing 3 mM $MgCl_2$ (Bio-Rad, Bio-Rad Laboratories Inc., Hercules, California, U.S.A.), 200 µM of each dNTP (Bio-Rad), 25 ρmol of each primer, 0.5 U of i*Taq*™ DNA polymerase (Bio-Rad), and 1 µl of gDNA (for phase 1) or 1 µl of purified amplicon (for phase 2). Amplicons (10 µl) were purified by adding 3 U of exonuclease I (New England BioLabs, Whitby, ON, Canada), 0.15 U of shrimp alkaline phosphatase (Fermentas, Fisher Scientific, Ottawa, ON, Canada), and 0.7 µl of 1X PCR buffer (Bio-Rad) prior to incubation at 37°C for 15 min and then 80°C for 15 min. PCRs were performed in a thermocycler (iCycler, Bio-Rad) using the following conditions; for the first phase: 95°C for 5 min, followed by 30 cycles of 95°C for 60 s, 58°C for 60 s, and 72°C for 60 s, and a final extension at 72°C for 5 min, and for the second phase: 95°C for 5 min, then 30 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 45 s, and a final extension at 72°C for 5 min. Negative (i.e., no gDNA) controls were included in each set of reactions. Amplicons (5 µl) were subjected to electrophoresis at 120 V for 40 min on 1.5% (w/v) agarose-TBE gels (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3; OmniPur®, EMD Millipore Ltd., Etobicoke, ON, Canada). A transilluminator emitting UV light was used to visualize the amplified DNA fragments.

The amplicons of seven PCR-positive samples were then purified using the methodology described in Chapter 2 and subjected to automated DNA sequencing at the National Research Council (Saskatoon, Saskatchewan) using the primers HGE1F and HGE3R in separate reactions. Sequence data were compared to the 16S rRNA sequences of the different strains of *A. phagocytophilum* available in GenBank™ using BLASTn® searches (National Center for

Biotechnology Information (NCBI); http://blast.ncbi.nlm.nih.gov). The sequences of the different strains were deposited in GenBank under the accession numbers HG916766-HG916767. The sites of restriction endonucleases within the 16S rRNA sequences of the Ap-ha and Ap-variant 1 strains were determined using the bioinformatics software DNASIS MAX (Version 3.00.000).

7.3.4. PCR-RFLP

A PCR-RFLP assay, developed (at the U of S) based on one of the nucleotide differences in 16S DNA sequence of the Ap-ha and Ap-variant 1 strains, was tested on the *A. phagocytophilum*-positive amplicons derived from the ticks collected in Minnesota. RFLP digests were performed in 30 µl volumes containing 15.5 µl of nuclease-free H_2O, 2.5 µl of the restriction enzyme *Kpn2I* (FastDigest®, Fermentas), 2 µl of 10x FastDigest Green Buffer (Fermentas), and 10 µl of unpurified PCR product from the second phase of the nPCR. Digests were performed at 37°C for 2 hrs and then at 80°C for 15 mins prior to loading (10 µl of product) on 1-2% (w/v) agarose-TBE gels that were subjected to electrophoresis for 1-2 hrs at 100 V. A nPCR was then conducted on the total gDNA of the 90 ticks collected from PVPP and ST (southern Manitoba), and the RFLP assay was tested on all PCR-positive samples. The undigested products of three PCR-positive samples from southern Manitoba were then purified and subjected to automated sequencing using primers HGE1F and HGE3R in separate reactions to determine that there was 100% concordance between RFLP pattern and strain type of *A. phagocytophilum*.

A modification of the PCR-RFLP assay was developed and tested (at NML) using amplicons produced by semi-nested PCR of the bacterial 16S rRNA gene. This modification was introduced, because the semi-nested PCR produced better quality amplicons from a few gDNA samples as compared to those produced by nPCR. In the first phase, primers Ge3a (5'-CACATG CAAGTCGAACGGATTATTC-3') and Ge10r (5'-TTCCGTTAAGAAGGATCTAATCTCC-3') (Massung et al. 1998) were used to amplify 932 bp of the 16S rRNA gene. Then, a smaller fragment (919 bp) was amplified using primers Ge9f (5'-AACGGATTATTCTTTATAGCTT GCT-3') (Massung et al. 1998) and Ge10r. All PCRs were conducted in 50 µl reaction volumes containing 3 mM $MgCl_2$ (Bio-Rad), 200 µM of each dNTP (Bio-Rad), 0.2 µM of each primer, 2.5 U of i*Taq* DNA polymerase (Bio-Rad), and 3 µl of gDNA (for phase 1) or 2 µl of unpurified

amplicon (for phase 2). PCRs were performed in a thermocycler (PTC-200 thermal cycler; MJ Research) using the following cycling conditions: 95°C for 2 min (phase 1) or 5 min (phase 2); followed by 40 cycles (for phase 1) or 30 cycles (for phase 2) of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s; and then 72°C for 5 min. The resulting 919 bp PCR-amplicons were digested with *Kpn2I* as described above. This PCR-RFLP assay was tested on the gDNA of 114 *A. phagocytophilum*-positive *I. scapularis* individuals (i.e., by *msp2* and 16S rRNA gene real-time PCRs) that were collected during the passive surveillance program from across Canada and 11 *A. phagocytophilum*-infected blacklegged ticks collected by drag sampling at two sites in Ontario. These 125 gDNA samples were selected based on their cycle threshold (Ct) value (i.e., ≤ 35) in the *msp2* real-time PCR assay. A subset (n = 58) of undigested amplicons were purified using Montage filter units (Millipore) and then subjected to automated DNA sequencing (on an ABI 3130x1 Genetic Analyzer using BigDye® Terminator and v3.1 cycle sequencing kits; Applied Biosystems®, Life Technologies Inc., Burlington, ON, Canada) using primers Ge9f and Ge10r in separate reactions to confirm 100% concordance between RFLP pattern and strain type of *A. phagocytophilum*. Sequence data were analyzed using DNASTAR Lasergene 9 software and compared to those in GenBank using BLASTn. The gDNA of all *A. phagocytophilum*-positive ticks from Minnesota previously tested by nPCR-RFLP at U of S were also subjected to the semi-nested PCR-RFLP assay (at NML) to test for concordance.

7.3.5. SNP assay

The 16S rDNA sequences of the Ap-ha and Ap-variant 1 strains were submitted to Applied Biosystems SNPbrowser to design a Custom TaqMan® SNP Genotyping Assay (P/N 4332077; Applied Biosystems). The SNP genotyping assay mixture contained two primers (Ap Forward: 5'-ACATGCAAGTCGAACGGATTATTCT-3' and Ap Reverse: 5'-GCTATCCCA TACTACTAGGTAGATTCCT-3') that flank the region (50 bp) containing two SNP sites (i.e., alignment positions 3 and 11 in Table 7.2), and two TaqMan probes, one that perfectly matched the Ap-ha strain (Ap-ha probe: 5'-CTGCCACTAACTATTCT-3') labelled with VIC, the other designed for the Ap-variant 1 strain (Ap-var 1 probe: 5'-CTGCCACTAATTATTCT-3') labelled with 6-carboxy-fluorescein (FAM). The probes contained a non-fluorescent quencher (NFQ) with a minor groove binder (MGB) at the 3' end. Binding of the MGB to the DNA template stabilizes the complex, allowing for shorter probe sequences. Real-time PCRs were performed

Table 7.2. Variable positions in the alignment of the 16S rRNA gene sequences of representative samples of *A*. *phagocytophilum* from the gDNA of seven *I*. *scapularis* (CS-F-21, CS-F-23, CS-F-24, CS-M-23, IS-F-4, IS-M-2 and CR-M-7) collected in Minnesota when compared to the sequences of the Ap-variant 1 and Ap-ha strains of *A*. *phagocytophilum* (Chen et al. 1994, Massung et al. 1998) from GenBank. A dot at an alignment position indicates the same nucleotide as in the 16S rRNA gene sequence of Ap-variant 1 strain (accession no. AY193887), while the * indicates an unknown nucleotide.

	Alignment position:		
	3	11	536
Ap-variant 1 strain (AY193887)	G	A	*
Ticks CS-F-21 & CR-M-7	.	.	A
Ap-ha strain (U02521)	A	G	G
Ticks CS-F-23, CS-F-24, CS-M-23, IS-F-4 & IS-M-2	A	G	G

using 96 well TaqMan Fast reaction plates (Applied Biosystems) in a 7500 Real-Time PCR System (Applied Biosystems). Reactions were carried out in a total volume of 25 µl that contained 12.5 µl of TaqMan Universal Master Mix (Applied Biosystems), 1.25 µl of 20X Custom TaqMan SNP Genotyping Assay (Applied Biosystems), 6.25 µl nuclease-free water, and 5 µl of gDNA or water (for the negative controls). The amplification conditions used were: 50°C for 2 min followed by 95°C for 10 min (for AmpliTaq Gold activation), and then 40 cycles of 95°C for 15 s and 60°C for 1 min. Detection was achieved through the 5' nuclease chemistry of AmpliTaq Gold polymerase, which cleaves an allele-specific 5' dye label and generates a permanent assay signal following the PCR process. The end-point data were analyzed to determine the distribution of variants of a single nucleic acid sequence by measuring the change in fluorescence of the probe-associated dyes. Analyses were performed by using the AB 7500 version 2.0.5 software. The strain discrimination plot was generated using the R_N (fluorescence) from the Ap-ha probe versus the R_N from the Ap-var 1 probe for a visual representation of the distribution of the *A. phagocytophilum* strains in the samples.

A representative sample of gDNA extracts of *I. scapularis* (n = 125) from the passive surveillance program confirmed to be positive for *A. phagocytophilum* by real-time PCR specific for the *msp2* and 16S rRNA genes and that were typed as either Ap-ha or Ap-variant 1 strain by PCR-RFLP were used to validate the SNP genotyping assay. Extracts were selected to represent samples from across the geographical range of the tick submissions, over several different years, and over a range of Ct values. In addition, the 17 *A. phagocytophilum*-infected *I. scapularis* ticks collected from Minnesota and the *A. phagocytophilum* equine isolate MN-93 were also subjected to the SNP analyses.

7.4. Results

7.4.1. Prevalence of *A. phagocytophilum*-infected *I. scapularis* in Canada

The proportion of *I. scapularis* individuals collected each year that were PCR-positive for *A. phagocytophilum* (Table 7.1) varied from 1-2.2% (average = 1.3%). There was no significant difference in the proportion of blacklegged ticks infected with *A. phagocytophilum* collected in 2007 through 2009 ($\chi^2_2 = 0.21$, $p > 0.05$), however there was a significant increase in number of infected ticks collected in 2010 ($\chi^2_1 = 23.86$, $p < 0.05$). There was no significant difference

($\chi^2_3 = 4.97$, $p > 0.05$) in the proportions of *A. phagocytophilum*-infected *I. scapularis* individuals collected from Quebec, New Brunswick, Nova Scotia, and Prince Edward Island over the four year period (i.e., with samples sizes >350). In contrast to the prevalence data from these provinces (1.5-2.8%), significantly fewer ticks from Ontario (0.3%), and significantly more from Manitoba (5.6%), were infected with *A. phagocytophilum* ($\chi^2_5 = 129.7$, $p < 0.001$).

7.4.2. Comparison of the 16S rRNA sequences of the Ap-ha and Ap-variant 1 strains

A single band of the expected size (~920 bp) for the partial 16S rRNA gene of *A. phagocytophilum* was detected on agarose gels for amplicons derived from the total gDNA of 17 (12 females and 5 males) of the 168 *I. scapularis* collected in Minnesota. A subset ($n = 7$) of amplicons were selected for DNA sequencing. The 870 bp sequences of five amplicons were identical to the 16S rRNA gene sequence of the Ap-ha strain (accession no. U02521) (Table 7.2). The sequences (870 bp) of the two other amplicons differed at three nucleotide positions (alignment positions 3, 11, and 536; Table 7.2) when compared to the sequence of the Ap-ha strain. The first 375 bp of these amplicons were identical in sequence to the 16S rDNA sequence of the Ap-variant 1 strain (accession no. AY193887), however it was not possible to compare the samples over the 870 bp because there are no sequence data available for this part of the 16S rRNA gene for the Ap-variant 1 strain. A comparison of the sequence alignments of the two strain types revealed nucleotide differences at three positions (Table 7.2). The mutational difference at alignment position 536 was associated with a restriction site for the endonuclease *Kpn2I* (T/CCGGA) in the sequence of the Ap-variant 1 strain that was absent in the sequence of the Ap-ha strain.

7.4.3. PCR-RFLP

Digestion of the 17 amplicons derived by nPCR (i.e., using primer combinations EC12A/EC9 and HGE1F/HGE3R) from the *A. phagocytophilum*-infected ticks collected in Minnesota with the endonuclease *Kpn2I* produced three different RFLP patterns on an agarose gel (Fig. 7.1). Three amplicons each had two bands (~360 and ~560 bp), while 13 amplicons remained undigested (i.e., a single band of ~920 bp), representing the expected patterns for the Ap-variant 1 strain and Ap-ha strain, respectively. These results were confirmed based on comparisons of the DNA sequences of representative samples. For one amplicon, three bands

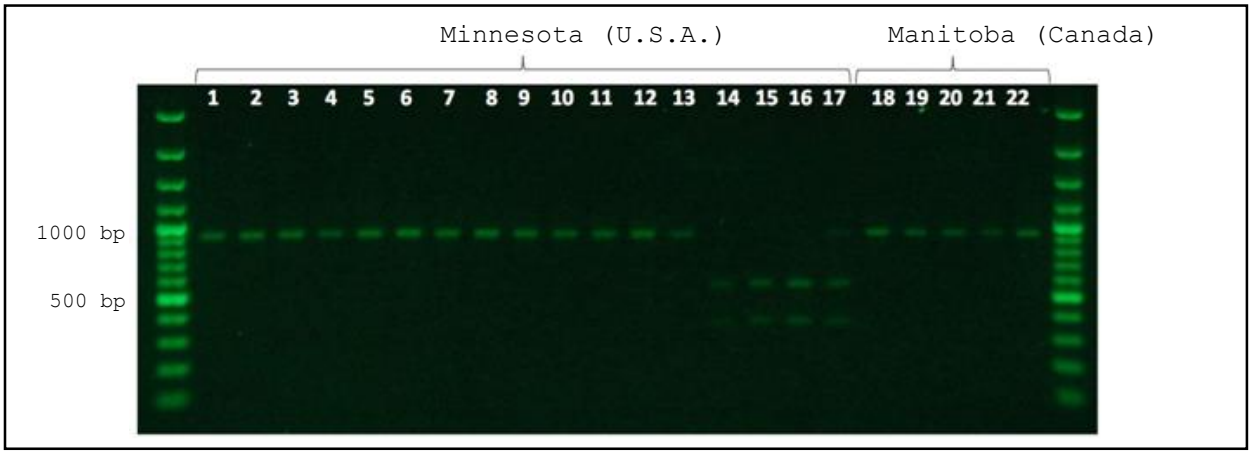


Figure 7.1. RFLP patterns of the 16S rDNA for 22 *A*. *phagocytophilum* PCR-positive *I*. *scapularis* collected from Minnesota (U.S.A.) and Manitoba (Canada). Notice the sample in lane 17 is a mixture of two strains.

were produced, suggesting a combination of the RFLP patterns for the Ap-ha strain (~920 bp) and Ap-variant 1 strain (~360 and ~560 bp) (Fig. 7.1). Of the 90 questing adult *I. scapularis* collected from PVPP and ST (southern Manitoba), five (3 females and 2 males) were PCR-positive for *A. phagocytophilum*, all of which had RFLP patterns consistent with those of the Ap-ha strain (Fig. 7.1). The presence of the gDNA of the Ap-ha strain in three ticks was confirmed by DNA sequencing.

The second RFLP analyses, based on digestion with *Kpn2I* of amplicons produced by semi-nested PCR (i.e., using primer combinations Ge3a/Ge10r and Ge9f/Ge10r), produced identical banding patterns for all samples from Minnesota (n = 17) tested using the first RFLP assay containing either the Ap-variant 1 strain (i.e., two bands of ~360 and ~560 bp), the Ap-ha strain (i.e., one band of ~920 bp), or a mixture of both strains (i.e., three bands of ~360, ~560, and ~920 bp) (not shown). Furthermore, RFLP analyses of the 125 *A. phagocytophilum*-infected ticks from across Canada revealed that 79 (63%) had a banding pattern consistent with the presence of the Ap-variant 1 strain, while the remaining 46 (37%) had a banding pattern indicating the presence of the Ap-ha strain. The DNA sequences of 58 samples, taken at random, showed 100% agreement between RFLP pattern and strain type of *A. phagocytophilum*.

7.4.4. SNP assay

The TaqMan SNP genotyping assay was used to determine the *A. phagocytophilum* strains present in the gDNA of infected *I. scapularis*. Ticks infected with different *A. phagocytophilum* strains could be easily distinguished from one another by their respective positions along either the Ap-ha probe axis or Ap-variant 1 probe axis on the discrimination plot (Fig. 7.2). As was expected, samples containing a lower concentration of *A. phagocytophilum* DNA, based on C_T values in real-time PCR, tended to cluster closer to the no template control on their respective axes. The SNP analyses revealed that of the 142 infected *I. scapularis* tested, 82 (58%) contained the Ap-variant 1 strain while 59 (42%) contained the Ap-ha strain, which was 100% in agreement with the results of the RFLP analyses. In addition, the SNP assay also clearly discriminated the tick from Itasca State Park (Minnesota) containing a mixture of both Ap-ha and Ap-variant 1 strains (as per PCR-RFLP analyses and DNA sequencing) from those ticks

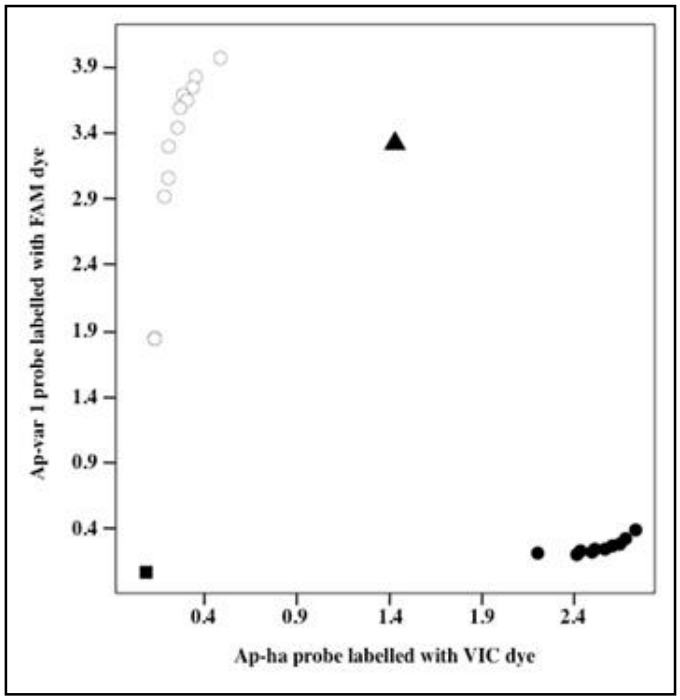


Figure 7.2. Allelic discrimination plot for the SNP assay based on the 16S rRNA gene of *A. phagocytophilum*. Closed circles represent samples that contain the Ap-ha strain while open circles represent samples that contain the Ap-variant 1 strain. The triangle represents a sample that contains a mixture of both strains. The solid square represents the no template control.

containing only one strain of *A. phagocytophilum* (Fig. 7.2). The results of the SNP analyses conducted on 125 ticks infected with *A. phagocytophilum* collected in different provinces by passive surveillance are shown in Table 7.3. There was no significant difference ($\chi^2_1 = 1.39$, $p > 0.05$) in the proportions of *I. scapularis* individuals from Quebec and Ontario infected with the Ap-ha strain. In contrast, a significantly ($\chi^2_2 = 40.48$, $p < 0.05$) lower proportion of ticks from these two provinces were infected with the Ap-ha strain when compared to those from the "western" region (i.e., Alberta, Saskatchewan, and Manitoba), and from three of the four Atlantic Provinces (i.e., Nova Scotia, New Brunswick, and Prince Edward Island).

7.5. Discussion

Human granulocytic anaplasmosis may represent an emerging infectious disease in southern Canada, because migratory passerine birds are transporting large numbers of the vector (*I. scapularis*) from the U.S.A. into the country each spring (Klich et al. 1996, Scott et al. 2001, Morshed et al. 2005, Ogden et al. 2008c, Scott et al. 2010, Scott et al. 2012), some of which are infected with *A. phagocytophilum* (Ogden et al. 2008c). In addition, blacklegged ticks collected from white-tailed deer culled in southern Quebec have also been recently shown to be infected with *A. phagocytophilum* (Bouchard et al. 2013). However, it was not determined if the blacklegged ticks on the migratory passerines or on white-tailed deer in these two studies (Ogden et al. 2008c, Bouchard et al. 2013) contained the human pathogenic (i.e., Ap-ha) strain of *A. phagocytophilum*. Furthermore, several populations of *I. scapularis* have recently become established or are in the process of establishing in southern Canada (Barker and Lindsay 2000, Ogden et al. 2008c, Ogden et al. 2008d, Ogden et al. 2009, Bouchard et al. 2011, Koffi et al. 2012), but little is known of the prevalence of *A. phagocytophilum* within blacklegged ticks from different geographical regions in southern Canada or of the relative proportions of ticks infected with the Ap-ha or Ap-variant 1 strains of the bacterium. The *msp2* PCR analyses conducted in the present study revealed that only a small proportion (~1%) of the ticks collected in Canada through the national passive tick surveillance program were infected with *A. phagocytophilum*. However, twice as many infected ticks were detected in 2010 than in the previous three years suggesting that there may have been an increase in prevalence of the bacterium in blacklegged ticks over time. Nonetheless, the prevalence of *A. phagocytophilum* in *I. scapularis* is lower than that reported for another human pathogen, *Borrelia burgdorferi*, the causative agent of Lyme

Table 7.3. The number of blacklegged ticks collected in different Canadian provinces in 2007 through 2010 that were infected with the Ap-ha or Ap-variant 1 strains of *A. phagocytophilum* using the TaqMan SNP genotyping assay.

Province	No.	Ap-ha (%)	Ap-variant 1 (%)
Western region			
Alberta	1	1 (100)	0 (0)
Saskatchewan	2	2 (100)	0 (0)
Manitoba	17	15 (88.8)	2 (11.8)
Central region			
Ontario	24*	2 (8.3)	22 (91.7)
Quebec	47	9 (19.2)	38 (80.8)
Atlantic region			
Prince Edward Island	7	3 (42.9)	4 (57.1)
New Brunswick	10	6 (60.0)	4 (40.0)
Nova Scotia	17	8 (47.1)	9 (52.9)
Total	125	46 (36.8)	79 (63.2)

* An additional 11 ticks were actively collected.

borreliosis, in *I. scapularis* from eastern Canada (Ogden et al. 2008c). It was also determined in the present study that there are significant differences in the prevalence of *A. phagocytophilum* in *I. scapularis* among the geographical regions in southern Canada. A greater proportion of blacklegged ticks in Manitoba were infected with *A. phagocytophilum* than in the other provinces, with the lowest proportion of uninfected ticks collected in Ontario. The proportion of all *I. scapularis* collected in Quebec in 2007 through 2010 that were infected with *A. phagocytophilum* (i.e., 1.5%) is considerably lower than that (i.e., 15%) reported for blacklegged ticks feeding on white-tailed deer culled in southern Quebec in 2007 and 2008 (Bouchard et al. 2013). However, this difference in prevalence may be due to the difference in sampling methods used in the two studies. In the study of Bouchard et al. (2013), there was a cluster of white-tailed deer in the centre of their study area that were parasitized by *I. scapularis*, a large proportion (77%) of which were infected with *A. phagocytophilum*.

Although knowledge of the prevalence of *A. phagocytophilum*-infected *I. scapularis* in different provinces in Canada provides some information for determining the public health risks for HGA, it does not take into account the relative frequency of the different strains of *A. phagocytophilum* present in blacklegged ticks. This is important to consider in risk assessment, since the Ap-ha strain, and not the Ap-variant 1 strain, has been associated with clinical cases of HGA (Massung et al. 2002). Therefore, there is a need for cost-effective and reliable assays to distinguish the human pathogenic strain of *A. phagocytophilum* from those not presently associated with human infection. The current method to delineate the Ap-ha strain from the Ap-variant 1 of *A. phagocytophilum* is to sequence amplicons of the partial 16S rRNA gene derived from the gDNA of infected blacklegged ticks (Massung et al. 1998, Massung et al. 2002, Courtney et al. 2003, Massung et al. 2003b, Massung et al. 2005, Michalski et al. 2006, Steiner et al. 2008).

Three PCR-based assays targeting the 16S rRNA gene of *A. phagocytophilum* were developed in the present study to provide additional methods to distinguish the Ap-ha strain from the Ap-variant 1 strain. Previous studies had reported two nucleotide differences at the 5' end of the 16S rRNA gene in the sequences of these two strains (Massung et al. 1998, Massung et al. 2002); however, a third nucleotide difference exists (i.e., at alignment position 536; Table 7.2) in the sequence alignment (870 bp) of the two strains. The mutational change at this third alignment position corresponds to the presence of a recognition site (T/CCGGA) for the endonuclease

Kpn2I in the sequence of the Ap-variant 1 strain that is absent in the sequence of the Ap-ha strain. Two RFLP assays using *Kpn2I* were developed at different laboratories, one based on restriction analyses of amplicons produced from a nPCR and the other on amplicons produced from a semi-nPCR, and tested on the same gDNA samples. There was 100% concordance in the RFLP patterns produced for all samples tested using the two RFLP assays. The reliability of the RFLP analyses were confirmed by comparing the DNA sequences of representative samples of each restriction pattern. In addition, the reliability of the PCR-RFLP assays was further demonstrated when the presence of both the Ap-ha and Ap-variant 1 strains were detected in the gDNA of one *I. scapularis* female (IS-F-17) collected from Itasca State Park in Minnesota. The occurrence of mixed infections of the Ap-ha and Ap-variant 1 strains in *I. scapularis* appears not to be common, and have been reported previously; for example, in a blacklegged tick from Bridgeport, Connecticut (Massung et al. 2002).

The presence of a mixed *A. phagocytophilum* infection in tick IS-F-17 was also detected using a TaqMan SNP genotyping assay we developed to distinguish the Ap-ha from the Ap-variant 1 strains. This SNP assay, based on a nucleotide difference (i.e., at alignment position 11; Table 7.2) at the 5' end of the 16S rRNA gene, was 100% effective in distinguishing these two strains of *A. phagocytophilum* because there was total concordance with the results of the PCR-RFLP and DNA sequencing analyses. Real-time PCR systems (e.g., TaqMan) have been shown to be sensitive and accurate for the detection of many pathogens (Paiva-Cavalcanti et al. 2010, Liu et al. 2013) and eliminate the need for post-amplification manipulations and the technical problems that are sometimes associated with RFLP analyses of amplicons produced by nPCR (Campsall et al. 2004, Kim et al. 2013). Moreover, the SNP assay is less technically demanding and more rapid than conventional nPCR-RFLP and DNA sequencing analyses, which take on average, two working days to complete. The formation of PCR product using the TaqMan system is monitored by real-time measurement of reporter dye fluorescence and analysis of amplification can be done within minutes of thermocycling completion with the SDS software. Therefore, the TaqMan method is ideal for clinical and epidemiological use where it may be essential to distinguish between the two strains of *A. phagocytophilum* in *I. scapularis* to assess the potential risk of human infection. Nonetheless, the 100% concordance in the results of the SNP, PCR-RFLP, and DNA sequence analyses in the present study suggest that the PCR-RFLP assays provide a reliable and cost-effective approach to distinguish the Ap-ha strain from the Ap-

variant 1 strain of *A. phagocytophilum* and will be particularly useful in research laboratories that lack the capacity to conduct real-time PCR. The PCR-RFLP assays also provide an independent and relatively inexpensive method to confirm the results obtained using the TaqMan SNP genotyping assay.

The PCR-based assays were used to determine the frequency of the Ap-ha or the Ap-variant 1 strains in *I. scapularis* from the different regions of southern Canada. Of the 125 *A. phagocytophilum*-infected ticks tested, 37% contained the Ap-ha strain, while the remaining 63% had the Ap-variant 1 strain. There were, however, significant differences in the frequency of blacklegged ticks infected with the Ap-ha strain among the regions in southern Canada. In southern Manitoba, where the greatest prevalence of *A. phagocytophilum* in *I. scapularis* was recorded, a large proportion (88%) of infected ticks carried the Ap-ha strain. In contrast, in southern Ontario and Quebec, very few *A. phagocytophilum*-infected *I. scapularis* (8 and 19%, respectively) contained the Ap-ha strain. Therefore, in addition to the geographical differences in the proportion of blacklegged ticks infected with *A. phagocytophilum*, there are significant geographical differences in the proportions of *I. scapularis* infected with the human pathogenic strain of *A. phagocytophilum*. Thus, the risk of exposure to this strain of *A. phagocytophilum* for Canadians appears to vary among geographical regions.

In summary, we have developed simple, rapid, effective and reliable PCR-based methods to discriminate the human pathogenic strain of *A. phagocytophilum* (Ap-ha) from the non-pathogenic strains (Ap-variant 1) in blacklegged ticks using the 16S bacterial rRNA gene as a genetic marker. Further work is needed to determine if any other variant strains, such as Ap-variant 2, which has only been reported from Rhode Island (Massung et al. 2002), that occur in *I. scapularis* and that are not associated with human disease can also be distinguished from the Ap-ha strain based on one or more of the assays described in the present paper. The findings of our study also show that the human pathogenic strain of *A. phagocytophilum* is present in questing *I. scapularis* adults in southern Canada, but its prevalence differs among geographical regions, suggesting differences in the potential risk of exposure to humans to this disease-causing bacterium. We therefore conclude that HGA represents a potential emerging disease in Canada and that additional studies are needed to monitor the prevalence of the Ap-ha strain in *I. scapularis* within different regions of southern Canada.

CHAPTER 8
PREVALENCE AND GENETIC DIVERSITY OF *ANAPLASMA PHAGOCYTOPHILUM* IN *IXODES SCAPULARIS*

8.1. Abstract

Phylogeographical relationships among strains of *Anaplasma phagocytophilum* infecting blacklegged ticks, *Ixodes scapularis*, were explored by comparing their DNA and amino acid sequences of the ankyrin (*ankA*) gene. Nested PCR analyses targeting the 16S ribosomal (r) RNA gene were also used to determine the prevalence of *A. phagocytophilum* infection in 513 *I. scapularis* collected from nine established populations. PCR-RFLP, SNP, and DNA sequence analyses of the 16S amplicons were conducted to determine if either the Ap-ha (i.e., human pathogenic) or the Ap-variant 1 (i.e., not associated with human infection) strain of *A. phagocytophilum* was present. The results showed that the proportion of ticks infected with the Ap-ha strain was significantly higher in the "western" geographical region than in the "central" and "eastern/Atlantic" regions. Sequence analyses of 76 *A. phagocytophilum*-positive ticks revealed the presence of 13 *ankA* strains that differed from one another by 1-43 bp. The corresponding AnkA amino acid sequences differed from one another by 1-35 aa. There were 54 and 41 variable positions in the 13 aligned DNA and amino acid sequences, respectively. Phylogenetic and phylogeographical analyses of the nucleotide (and corresponding amino acid) sequences of *ankA* revealed the existence of three clades of *A. phagocytophilum*: Clade 1 contained only Ap-variant 1 strains based on sequence analyses of the 16S rRNA gene, while Clades 2 and 3 contained only Ap-ha strains. Clades 1 and 2 comprised strains detected in both the "western" and "eastern" regions of North America, whereas Clade 3 contained strains only found in the "eastern" region. All *A. phagocytophilum* defined as the Ap-ha strain did not represent a monophyletic assemblage based on phylogenetic analyses of the nucleotide (and corresponding amino acid) sequences of *ankA*. The biological and evolutionary significance of these findings are discussed.

8.2. Introduction

Anaplasma phagocytophilum, previously known as *Ehrlichia phagocytophila*, *Ehrlichia equi*, and the "HGE agent," is a gram-negative bacterium within the family Anaplasmataceae and

the order Rickettsiales (Roux and Raoult 1995, Dumler et al. 2001). This non-motile, obligate, intracellular bacterium is a tick-borne pathogen of humans, domestic animals (e.g., dogs, cats, horses, sheep, goats, and cattle), and wildlife (e.g., white-footed mice, voles, shrews, gray squirrels, chipmunks, wild boars, red foxes, bears, bison, roe deer, red deer, and white-tailed deer) (Foley et al. 2004, Foley et al. 2008a, Ismail et al. 2010, Vichova et al. 2010, Scharf et al. 2011, Jin et al. 2012, Huhn et al. 2014) in Europe (Oteo et al. 2000, Lotrič-Furlan et al. 2006, Mastrandrea et al. 2006, Foley et al. 2008a, Rymaszewska 2010, Vichova et al. 2010, Scharf et al. 2011, Huhn et al. 2014), Asia (Cao et al. 2003, Cao et al. 2006, Foley et al. 2008a, Zhan et al. 2008, Wuritu et al. 2009, Zhan et al. 2010, Ybanez et al. 2012b) and North America (Bakken et al. 1994, Massung et al. 2000, Holman et al. 2004, Massung et al. 2005, Poitout et al. 2005, Steiner et al. 2006, Yabsley et al. 2006, Foley et al. 2008a, Foley et al. 2008b, Foley et al. 2008c, Foley et al. 2008d, Steiner et al. 2008, Baldridge et al. 2009, Cockwill et al. 2009, Gabriel et al. 2009, Granick et al. 2009, Nieto et al. 2009, Nieto et al. 2010, Uehlinger et al. 2011, Burgess et al. 2012, Clark 2012, Dibernardo et al. 2014, Keesing et al. 2014, Prusinski et al. 2014). Tick species known to carry *A. phagocytophilum* include: *Ixodes persulcatus, Ixodes ovatus*, and *Haemaphysalis douglasii* in Asia (Cao et al. 2003, Wuritu et al. 2009, Ybanez et al. 2012b); and *Ixodes ricinus* and *I. trianguliceps* in Europe (von Loewenich et al. 2003a, Chmielewska-Badora et al. 2007, Bown et al. 2009). This pathogenic bacterium is also vectored by several species of tick in the U.S.A., such as: *Dermacentor albipictus* in the Midwest (Baldridge et al. 2009); *Ixodes pacificus*, *Ixodes angustus*, *Ixodes ochotonae*, *Ixodes spinipalpis*, and *Ixodes woodi* along the West Coast (Rejmanek et al. 2013); and *Ixodes dentatus* (Goethert and Telford 2003) and *Ixodes scapularis* (i.e., the blacklegged tick) in the Midwest and Northeast (Pancholi et al. 1995, Levin et al. 2002, Massung et al. 2002, Courtney et al. 2003, Goethert and Telford 2003, Massung et al. 2003a, Massung et al. 2003b, Holman et al. 2004, Lovrich et al. 2011, Keesing et al. 2014, Prusinski et al. 2014). In Canada, the bacterium has been detected in *I. scapularis* and *I. dentatus* (Drebot et al. 2001, Ogden et al. 2008c, Bouchard et al. 2013, Dibernardo et al. 2014, Krakowetz et al. 2014).

There are differences in the prevalence of *A. phagocytophilum*-infected *I. scapularis* among geographical regions in North America (Telford et al. 1996, Massung et al. 2002, Courtney et al. 2003, Shukla et al. 2003, Michalski et al. 2006, Steiner et al. 2008, Krakowetz et al. 2014). In the U.S.A., its prevalence in questing ticks (i.e., ticks collected from vegetation)

ranges from 4-14% in the Midwest (Shukla et al. 2003, Michalski et al. 2006, Steiner et al. 2008) to 1-23% in the Northeast (Telford et al. 1996, Massung et al. 2002, Courtney et al. 2003, Steiner et al. 2008). In Canada, the prevalence of *A. phagocytophilum* in blacklegged ticks also varies among geographical regions (Dibernardo et al. 2014, Krakowetz et al. 2014). There are two common strains of *A. phagocytophilum* that occur in blacklegged ticks (Massung et al. 1998, Drebot et al. 2001, Layfield and Guilfoile 2002, Massung et al. 2002, Courtney et al. 2003, Massung et al. 2003a, Massung et al. 2003b, Massung et al. 2005, Michalski et al. 2006, Steiner et al. 2008, Krakowetz et al. 2014), the human pathogenic strain (i.e., Ap-ha) (Chen et al. 1994, Lovrich et al. 2011) and a strain (i.e., Ap-variant 1) that has not been associated with human infection (Massung et al. 1998). These two strains are distinguished based on sequence differences in the 16S ribosomal (r) RNA gene (Massung et al. 1998, Krakowetz et al. 2014). There are differences in the proportions of these two strains in different parts of the distributional range of *I. scapularis* (Massung et al. 1998, Layfield and Guilfoile 2002, Massung et al. 2002, Courtney et al. 2003, Massung et al. 2005, Michalski et al. 2006, Steiner et al. 2008, Krakowetz et al. 2014).

Several additional markers have been used to characterize strains of *A. phagocytophilum*. These include sequences of the citrate synthase (*gltA*), ankyrin (*ankA*), and *groESL* operon genes. Major surface protein genes (e.g., *msp2*/*p44* and *msp4*) have also been used (Caturegli et al. 2000, Massung et al. 2000, von Loewenich et al. 2003a, de la Fuente et al. 2005, Cao et al. 2006, Shukla et al. 2007, Foley et al. 2008a, Zhan et al. 2008, Wuritu et al. 2009, Rymaszewska 2010, Zhan et al. 2010, Scharf et al. 2011, Clark 2012, Ybanez et al. 2012b). The *gltA* gene has been useful for resolving phylogenetic relationships among the rickettsiae (Roux et al. 1997, Billings et al. 1998, Inokuma et al. 2001, Heise et al. 2010, Anstead and Chilton 2013). However, little is known about whether this marker would be useful for examining the phylogeography of *A. phagocytophilum*, except that its strains form two clades corresponding to the U.S.A. and Eurasia (Cao et al. 2006, Shukla et al. 2007, Zhan et al. 2008, Zhan et al. 2010, Ybanez et al. 2012a). The *ankA* gene has also been used in several phylogenetic studies (Massung et al. 2000, von Loewenich et al. 2003a, Shukla et al. 2007, Foley et al. 2008a, Scharf et al. 2011). In some studies, the extent of the diversity in *ankA* of *A. phagocytophilum* has been found to be high (e.g., von Loewenich et al. 2003a). Furthermore, analysis of the different *ankA* strains has provided some phylogeographical information (Massung et al. 2000, von Loewenich

et al. 2003a, Shukla et al. 2007, Foley et al. 2008a). For example, there is evidence of two clades of *A. phagocytophilum* based on sequences of *ankA*: one in North America, and the other in Eurasia (Massung et al. 2000, von Loewenich et al. 2003a, Shukla et al. 2007, Foley et al. 2008a). Moreover, there may be additional clades present within each of these geographical areas, as there is some evidence to suggest that the North American clade consists of two groups: one representing the Northeast, U.S.A., and the other the Upper Midwest, U.S.A. (Massung et al. 2000, Foley et al. 2008a), and that the Eurasian clade consists of two groups: one corresponding to Europe, and the other to Asia (Foley et al. 2008a). However, little is known regarding the extent of the variability within this gene on a regional or local spatial scale and if there is an association between *ankA* strains of *A. phagocytophilum* and particular geographical regions (e.g., within North America).

Although there is some knowledge of the prevalence of *A. phagocytophilum* in adult *I. scapularis* collected from white-tailed deer in southeastern Ontario (ON) (<1% in 1999; Drebot et al. 2001) and southwestern Quebec (15% during 2007-2008; Bouchard et al. 2013), Canada, as well as in nymphal *I. scapularis* (<1% during spring in 2005 and 2006) collected from migratory birds at bird observatories across eastern Canada (Ogden et al. 2008c), there is no published information on the population genetics or phylogeography of *A. phagocytophilum* in Canada. In this chapter, I examine the diversity of *ankA* strains of *A. phagocytophilum* in *I. scapularis* from different geographical areas of Canada and the U.S.A. to test the hypothesis that there are geographical differences in the presence and/or prevalence of *A. phagocytophilum* strains.

8.3. Materials and Methods

8.3.1. Samples

A total of 573 adult and nymphal *I. scapularis* were collected from the environment or hosts (i.e., cats, dogs, and persons) for use in the present study (see Tables 2.1, 2.2, and Fig. 2.1). Of these, 513 *I. scapularis* were gathered from five resident populations in the U.S.A. (i.e., Itasca State Park, Camp Ripley (CR), and St. Croix State Park (CSP) in Minnesota, and Trustom Pond (TPSK) and Hazard Island (HISK) in South Kingstown, Rhode Island) and four resident populations in Canada (i.e., Pembina Valley Provincial Park (PVPP) and Stanley Trail (ST) in

Manitoba, and Point Pelee National Park (PPNP) and Long Point Provincial Park (LPPP) in Ontario). In addition to the 512 ticks from resident populations described in Chapter 2, an additional male *I. scapularis* individual, collected by drag-sampling the resident population in LPPP, was included in the analyses. The other 60 ticks were adventitious and collected as part of a national passive tick surveillance program conducted in Canada by the National Microbiology Laboratory (NML) in Winnipeg, Canada. All adventitious *I. scapularis* were obtained from hosts or the environment within nine Canadian provinces from Alberta to Newfoundland (NL).

8.3.2. Molecular analyses

The total genomic (g) DNA of each tick was extracted and purified using the materials and methodology described in Chapter 2. A portion of the *I. scapularis* (i.e., 258 ticks; 90 from MB and 168 from MN) was previously screened for *A. phagocytophilum* using nested PCR (nPCR) targeting the bacterial 16S rRNA gene, as part of the study described in Chapter 7. A second portion of the *I. scapularis* (i.e., the 60 adventitious ticks) was previously determined to be infected with *A. phagocytophilum* by real-time PCR targeting the *msp2* gene (Courtney et al. 2004) at the NML, as part of a national surveillance program. The presence of *A. phagocytophilum* was also confirmed at the NML for all 60 adventitious *I. scapularis* by real-time PCR targeting the 16S rRNA gene of *A. phagocytophilum*, as described in Chapter 7. At the University of Saskatchewan (U of S), nPCR was used to amplify part (~920 bp) of the 16S rRNA gene of *A. phagocytophilum* from these same 60 adventitious ticks. All nPCRs were conducted as described in Chapter 7, except that the annealing temperature for the first phase was increased to 62°C. The remaining portion of the *I. scapularis* (i.e., 255 ticks; 155 from Ontario and 100 from Rhode Island) was screened for *A. phagocytophilum* using these modified nPCR conditions targeting the 16S rRNA gene. Amplicons (5 µl) resulting from the second phase of the nPCR were subjected to electrophoresis at 120 V for 40 min on 1.5% (w/v) agarose-TBE gels (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3; OmniPur®, EMD Millipore Ltd., Etobicoke, ON, Canada). The amplified DNA fragments were visualized using a transilluminator emitting UV light. Purification of the amplicons (10 µl) was performed by adding 3 U of exonuclease I (New England BioLabs, Whitby, ON, Canada), 0.15 U of shrimp alkaline phosphatase (Fermentas, Fisher Scientific, Ottawa, ON, Canada), and 0.7 µl of 1X PCR buffer (Bio-Rad, Bio-Rad Laboratories Inc., Hercules, California, U.S.A.) prior to incubation in a thermocycler (iCycler®,

Bio-Rad) at 37°C for 15 min and then 80°C for 15 min. A subset of amplicons (i.e., seven from Minnesota, three from Manitoba, and 12 from Rhode Island) was selected for automated DNA sequencing. Sequencing of the DNA was completed at the National Research Council (NRC) (Saskatoon, Saskatchewan) using the primers HGE1F and HGE3R in separate reactions. Sequences were manually aligned and compared to one another, as well as to those available on GenBank™ using BLASTn® searches (National Center for Biotechnology Information (NCBI); http://blast.ncbi.nlm.nih.gov). The sequences of the different 16S rRNA gene strains of *A. phagocytophilum* were previously deposited in GenBank under the accession numbers HG916766-HG916767.

Samples that were PCR-positive for *A. phagocytophilum* using the PCR assays conducted at the U of S and NML targeting the 16S rRNA gene were confirmed using the primers ANK-F1 (5'-GAAGAAATTACAACTCCTGAAG-3') (Massung et al. 2007) and LA1 (5'-CGTTCA GCCATCATTGTGAC-3') (Chmielewska-Badora et al. 2007) in PCRs targeting *ankA* of *A. phagocytophilum*. These PCRs were conducted in 25 µl reaction volumes containing 1.5 mM $MgCl_2$ (Bio-Rad), 200 µM of each dNTP (Bio-Rad), 25 ρmol of each primer, 0.5 U of i*Taq*™ DNA polymerase (Bio-Rad), and 1-2 µl of gDNA. The thermocycler (iCycler, Bio-Rad) conditions for the reactions were as follows: the initial denaturation was conducted at 95°C for 5 min; subsequently, 35 cycles consisting of a 60 s denaturation at 94°C, a 60 s annealing at 55°C, and a 60 s extension at 72°C were performed; the final extension was run at 72°C for 5 min. Amplicons were loaded onto 1.5% (w/v) agarose-TBE gels and subjected to electrophoresis at 120 V for 30 min or 130 V for 35 min. The size of the amplified DNA fragment, including the primers, was expected to be ~890 bp. Amplicons were then purified, using the same components and protocol as was used for the 16S rRNA gene (described above), prior to being subjected to automated DNA sequencing using the primers ANK-F1 and LA1 in separate reactions at the NRC.

The primer pairs: ANK-F2 (5'-TTGACCGCTGAAGCACTAAC-3') (Massung et al. 2007) and LA1, as well as LA6 (5'-GAGAGATGCTTATGGTAAGAC-3') (Chmielewska-Badora et al. 2007) and LA1, were also used to amplify part of the *ankA* gene of *A. phagocytophilum* by PCR. These additional PCR assays were conducted in order to provide confidence that the sequences obtained using the ANK-F1/LA1 primer pair accurately represented each sample. Therefore, only a subset of the *A. phagocytophilum*-containing samples

based on PCR targeting the 16S rRNA gene of the bacterium was tested using each primer pair (i.e., ANK-F2/LA1 and LA6/LA1). All of the *A. phagocytophilum*-containing samples from Minnesota (i.e., 17 samples) and Manitoba (i.e., five samples) were subjected to PCR in combination with the ANK-F2/LA1 primer pair, whereas only four samples from Minnesota were tested with LA6/LA1. Two of the four samples subjected to PCR in combination with the LA6/LA1 primer pair represented the Ap-ha strain of *A. phagocytophilum* based on analyses of the 16S rRNA gene, while another represented the Ap-variant 1 strain. The remaining sample represented a mixture of the Ap-ha and Ap-variant 1 strains. PCRs containing the ANK-F2 and LA1 primers were conducted in 25 µl reaction volumes using the same reagents in the same concentrations as described for the ANK-F1/LA1 primer pair, except that 1 µl of gDNA was consistently used. The following thermocycler conditions were used for the amplifications: the initial denaturation took place over 5 min at 95°C; then, 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s were performed; lastly, the final extension was run at 72°C for 5 min. The expected size of the amplified DNA fragment, including both primers, was ~850 bp. PCRs with the LA6 and LA1 primers were also conducted in 25 µl reaction volumes using the same reagents in the same concentrations as described for the ANK-F1/LA1 primer pair, except that 3 mM $MgCl_2$ and 1-1.5 µl of gDNA was used. The amplifications were carried out using the following thermocycler conditions: 30 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s, preceded by a 5 min denaturation at 95°C and followed by a 5 min extension at 72°C. The amplified DNA fragment, including the primers, was expected to be ~450 bp. Amplicons resulting from the different primer pairs were loaded onto separate 1.5% (w/v) agarose-TBE gels and electrophoresed at 120 V for 45 min (ANK-F2/LA1) or 35 min (LA6/LA1). A UV-emitting transilluminator was used to visualize the amplified DNA fragments. Purification of the amplicons (10 µl) was then performed, as described above for the 16S rRNA gene, prior to automated DNA sequencing, which was conducted at the NRC using the primers ANK-F2 and LA1 with the ~850 bp DNA fragments in separate reactions, as well as LA6 and LA1 with the ~450 bp DNA fragments in separate reactions. The DNA sequences were manually aligned and compared to one another in addition to those associated with the ANK-F1/LA1 primer pair. Comparisons were also made between the resultant DNA sequences and those available on GenBank using BLASTn searches (NCBI).

The 16S rRNA gene strains of *A. phagocytophilum* were also determined using restriction fragment length polymorphism (RFLP) analysis and single nucleotide polymorphism (SNP) genotyping for some ticks, as previously described (Chapter 7). At the U of S, a RFLP analysis was conducted on the five samples from Manitoba that were PCR-positive for *A. phagocyophilum*, as well as the 17 samples from Minnesota that were found to contain the bacterium by PCR (Chapter 7). At the NML, both RFLP and SNP analyses were used to confirm the strains of *A. phagocytophilum* infecting the 17 *I. scapularis* collected from Minnesota (Chapter 7). In the present study, the strains of *A. phagocytophilum* infecting the 60 adventitious *I. scapularis* were also determined, as previously described (Chapter 7), using SNP analyses at the NML. Also performed at the NML, as previously described (Chapter 7), were RFLP assays on a subset of the *A. phagocytophilum*-containing samples (n = 38) derived from the adventitious *I. scapularis* in order to validate the results of the SNP assay.

8.3.3. Data analyses

Phylogenetic analyses of the *ankA* sequence data were conducted using the neighbour-joining (NJ) and maximum parsimony (MP) methods, as implemented in the computer program PAUP v4.0b10 (Swofford 2002). A sequence (GenBank accession no. AY282377) obtained from an arbitrary *Ixodes ricinus* individual collected in Germany was used to root the trees (von Loewenich et al. 2003). Bootstrap analyses (1,000 and 100 replicates for NJ and MP, respectively) were used to evaluate the robustness of branches in the resulting trees. Relationships among the *ankA* sequences were also depicted in a minimum spanning network, which was generated using the computer program TCS version 1.21 (Clement et al. 2000). Included in these analyses were some of the *ankA* sequences reported in other studies (Storey et al. 1998, Pusterla et al. 1999, Caturegli et al. 2000, Massung et al. 2000, Lodes et al. 2001, Von Loewenich et al. 2003b, Dunning Hotopp et al. 2006, Massung et al. 2007, Domingos et al. 2011, Scharf et al. 2011, Katargina et al. 2012, Henniger et al. 2013) and/or deposited on GenBank (Table 8.1). The *ankA* DNA sequences (i.e., CK1-CK13, as well as GenBank accession nos. AF100882 and AF153716) were translated into amino acid (AnkA) sequences using the ExPASy Bioinformatic Resource Portal (http://web.expasy.org/translate/). A phylogenetic analysis (NJ) was conducted on the corresponding AnkA sequences using the methodology described above, except that the resulting tree was unrooted. The number of "fixed differences" in DNA and

Table 8.1. Strain/isolate names, host species, and geographical collection sites in North America and Europe corresponding to the *ankA* sequences (i.e., DNA sequences) of *A. phagocytophilum* that were obtained from GenBank and different studies and that were used in the phylogenetic and phylogeographical analyses.

GenBank accession no.	Strain/Isolate (str/iso)	Host	State/Province	Source
U.S.A.				
DQ320650	str Ap-variant 1 iso MN-29-1	tick (*Ixodes scapularis*)	Minnesota	Massung et al. 2007
DQ320651	str Ap-variant 1 iso MN-62-6	tick (*I. scapularis*)	Minnesota	Massung et al. 2007
DQ320652	str Ap-variant 1 iso MN-61-2	tick (*I. scapularis*)	Minnesota	Massung et al. 2007
DQ320653	str Ap-variant 1 iso MN-38	tick (*I. scapularis*)	Minnesota	Massung et al. 2007
DQ320654	str Ap-variant 1 iso MN-53-1	tick (*I. scapularis*)	Minnesota	Massung et al. 2007
AF100890	WI1 (HGE agent)	human (*Homo sapiens*)	Wisconsin	Massung et al. 2000
AF100891	WI2 (HGE agent)	human (*H. sapiens*)	Wisconsin	Massung et al. 2000
AF100892	WI3 (HGE agent)	human (*H. sapiens*)	Wisconsin	Massung et al. 2000
AF100893	WI4 (HGE agent)	human (*H. sapiens*)	Wisconsin	Massung et al. 2000
AF356512	iso WI 1	human (*H. sapiens*)	Wisconsin	Lodes et al. 2001
AF100883	NY1 (HGE agent)	human (*H. sapiens*)	New York	Massung et al. 2000
AF100884	NY2 (HGE agent)	human (*H. sapiens*)	New York	Massung et al. 2000
AF100885	NY3 (HGE agent)	human (*H. sapiens*)	New York	Massung et al. 2000
CP000235	str HZ	human (*H. sapiens*)	New York	Dunning Hotopp et al. 2006
AF047897	str BDS/*hge-d* (HGE agent)	human (*H. sapiens*)	unavailable	Caturegli et al. 2000
GU236806	iso human_03HE	human (*H. sapiens*)	unavailable	Scharf et al. 2011
GU236807	iso human_96HE54	human (*H. sapiens*)	unavailable	Scharf et al. 2011
GU236808	iso human_96HE58	human (*H. sapiens*)	unavailable	Scharf et al. 2011
AF020521	str USG3	dog (*Canis lupus familiaris*) which was experimentally infected using adult ticks (*I. scapularis*)	unavailable / New York & Pennsylvania	Storey et al. 1998
AF100894	MN-dog (CGE agent)	dog (*C. lupus familiaris*)	Minnesota	Massung et al. 2000
DQ680013	unavailable	cat (Felis catus)	North Carolina	Diniz et al., Unpublished
AF100882	EE	horse	California	Massung et al. 2000
AF153716	str MRK/*ee-o*	horse	California	Caturegli et al. 2000
DQ320648	str Ap-variant 1 iso RI-1	goat	Rhode Island	Massung et al. 2007
DQ320649	str Ap-variant 1 iso RI-2	goat	Rhode Island	Massung et al. 2007
Austria				
GU236826	iso dog_16	dog (*C. lupus familiaris*)	---	Scharf et al. 2011
GU236864	iso cat_1	cat (*F. catus*)	---	Scharf et al. 2011
Belarus				
HQ629927	str Bel11-2-07	tick (*Ixodes ricinus*)	---	Katargina et al. 2012
HQ629929	str Bel6-22-07	tick (*I. ricinus*)	---	Katargina et al. 2012
HQ629930	str BelBmi11	tick (*I. ricinus*)	---	Katargina et al. 2012
Estonia				
HQ629928	str Est3238	tick (*I. ricinus*)	---	Katargina et al. 2012
France				
GU391597	str Violetti	dog (*C. lupus familiaris*)	---	Domingos et al. 2011
GU391598	str Rodier	dog (*C. lupus familiaris*)	---	Domingos et al. 2011

Table 8.1. Continued.

GenBank accession no.	Strain/Isolate (str/iso)	Host	State/Province	Source
Germany				
AY282377	iso I68	tick (*I. ricinus*)	---	von Loewenich et al. 2003
AY282388	iso W37	tick (*I. ricinus*)	---	von Loewenich et al. 2003
AY282391	iso X7	tick (*I. ricinus*)	---	von Loewenich et al. 2003
GU236841	iso dog_34	dog (*C. lupus familiaris*)	---	Scharf et al. 2011
GU236843	iso dog_36	dog (*C. lupus familiaris*)	---	Scharf et al. 2011
GU236851	iso dog_54	dog (*C. lupus familiaris*)	---	Scharf et al. 2011
AF482759	unavailable	horse	---	von Loewenich et al. 2003
GU236857	iso horse_4_FR	horse (*Equus caballus*)	---	Scharf et al. 2011
GU236863	iso horse_S3041_06	horse (*E. caballus*)	---	Scharf et al. 2011
KC740482	iso cow_A262	cow (*Bos taurus*)	---	von Loewenich, unpublished
KC776919	iso calf_T633_2 clust I	cow (*B. taurus*)	---	Henniger et al. 2013
Norway				
GU236731	iso cow_355_04	cow (*B. taurus*)	---	Scharf et al. 2011
GU236755	iso sheep_adult1_KK	sheep (*Ovis aries*)	---	Scharf et al. 2011
GU236799	iso sheep_6054	sheep (*O. aries*)	---	Scharf et al. 2011
Poland				
GU236735	iso bison_8	bison (*Bison bonasus*)	---	Scharf et al. 2011
Russia				
HQ629926	str Rus30-13	tick (*I. ricinus*)	---	Katargina et al. 2012
Slovenia				
AF100886	Sl-HG1 (HGE agent)	human (*H. sapiens*)	---	Massung et al. 2000
AF100887	Sl-HG2 (HGE agent)	human (*H. sapiens*)	---	Massung et al. 2000
GU236802	iso human_2118	human (*H. sapiens*)	---	Scharf et al. 2011
GU236805	iso human_6219	human (*H. sapiens*)	---	Scharf et al. 2011
GU236718	iso red_deer_473	red deer (*Cervus elaphus*)	---	Scharf et al. 2011
GU236722	iso red_deer_812	red deer (*C. elaphus*)	---	Scharf et al. 2011
Spain				
GU236729	iso cow_2513_656	cow (*B. taurus*)	---	Scharf et al. 2011
Sweden				
AF100888	Sw-HG (HGE agent)	human (*H. sapiens*)	---	Massung et al. 2000
AY529487	str Susy	horse	---	Franzen et al., unpublished
AY529488	str Strong	horse	---	Franzen et al., unpublished
AF100889	EP	cow	---	Massung et al. 2000

--- not determined.

amino acid sequences (i.e., where all members of one clade have a different nucleotide or amino acid when compared to all members of another clade) were also determined.

8.4. Results

8.4.1. Molecular analyses and genetic diversity of the different 16S rRNA gene strains of *A. phagocytophilum*

A single band of the expected size (~920 bp) was detected on agarose gels for amplicons of the 16S rRNA gene of *A. phagocytophilum* derived from the total gDNA of 94 *I. scapularis* individuals (i.e., 60 adventitious ticks and 34 from established populations) (Fig. 8.1). No bands were detected for the negative control samples. Of the 513 *I. scapularis* screened for *A. phagocytophilum* by PCR targeting the 16S rRNA gene, 34 (6.6%) were PCR-positive (Table 8.2). These corresponded to 22 adults and 12 nymphs. Although approximately twice as many females (15 ticks) were PCR-positive for *A. phagocytophilum*, as compared to males (7 ticks), there was no significant difference among sexes in the proportion infected with *A. phagocytophilum* ($\chi^2_1 = 2.49$, $p > 0.05$).

Alignment of the DNA sequences (870 bp) of a portion of the 16S rRNA gene of *A. phagocytophilum* revealed the presence of two strains, which differed at three nucleotide positions. The DNA sequences of these two strains were identical to the 16S rRNA gene sequence of the Ap-ha strain (GenBank accession nos. U02521 and HG916766) and Ap-variant 1 strain (GenBank accession nos. AY193887 and HG916767). It was possible to determine whether an individual tick was infected with the Ap-ha, Ap-variant 1, or both the Ap-ha and Ap-variant 1 strains of *A. phagocytophilum* using the SNP assay. The RFLP analyses revealed that there were three different banding patterns among amplicons obtained from 60 gDNA samples (i.e., 38 adventitious ticks and 22 from established populations) (Fig. 8.2). Thirty-four samples had a single (undigested) band of ~920 bp consistent with the expected pattern for the Ap-ha strain. Another 25 samples each had two bands (~360 and ~560 bp) consistent with the expected pattern for the Ap-variant 1 strain. The third pattern was comprised of three bands (~360, ~560, and ~920 bp) and represented a mixture of both strains in a single tick. The different molecular analyses (i.e., DNA sequencing, SNP, and RFLP) were 100% concordant. The Ap-ha strain of *A. phagocytophilum* was detected in 56.4% of the 94 adult and nymphal *I. scapularis* that were

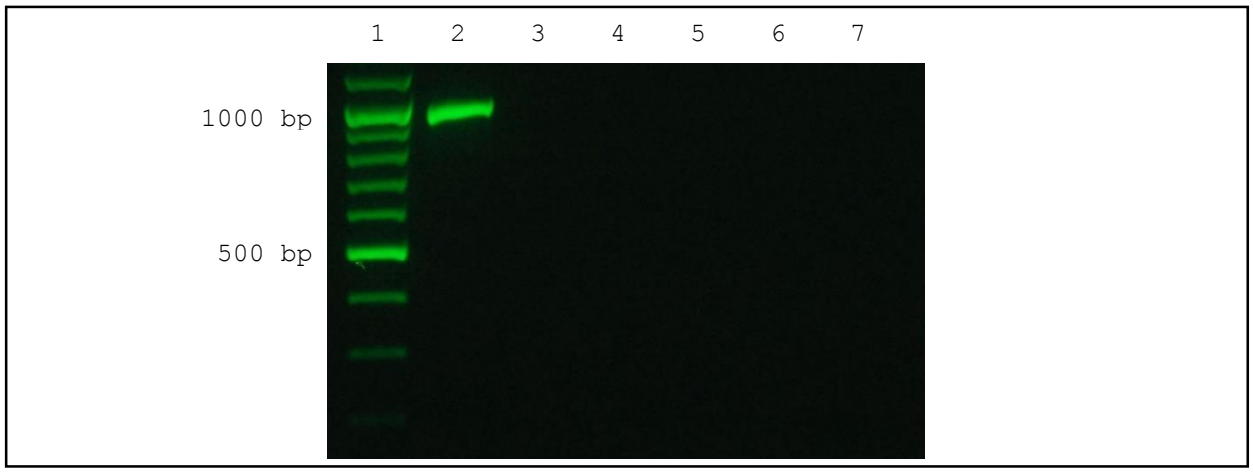


Figure 8.1. Agarose gel showing that the size of the amplicons obtained via nested PCR of the 16S rRNA gene of *A. phagocytophilum* was consistent with that which was expected (~920 bp). Lane 1 contains a standard DNA ladder (GeneRuler™ 100 bp Plus DNA Ladder 100 to 3000 bp, Thermo Fisher Scientific, Ottawa, ON, Canada). Lane 2 contains amplicons of the 16S rRNA gene of *A. phagocytophilum* from a sample collected in St. Croix State Park, Minnesota, U.S.A. Amplicons were absent in lanes 3-5, indicating that the bacterium was not present in these tick samples (or that the DNA of the bacterium was present, but could not be detected). Lanes 6 and 7 correspond to the negative controls of phases 1 and 2 of the nPCR, in which amplicons were not detected.

Table 8.2. The number and proportion (%) of *A. phagocytophilum*-infected *I. scapularis*, as determined by PCR targeting the 16S rRNA gene, in the nine established populations.

Country / Province/State / Locality*	Life cycle stage:											
	Nymphs:			Males:			Females:			Total:		
	n	PCR-positive	(%)	*n*	PCR-positive	(%)	*n*	PCR-positive	(%)	*n*	PCR-positive	(%)
Canada												
Manitoba												
PVPP	0	-		25	2	(8.0)	21	3	(14.3)	46	5	(10.9)
ST	0	-		25	0	(0.0)	19	0	(0.0)	44	0	(0.0)
Total	0	-		50	2	(4.0)	40	3	(7.5)	90	5	(5.6)
Ontario												
PPNP	0	-		0	-		46	0	(0.0)	46	0	(0.0)
LPPP	0	-		65	0	(0.0)	44	0	(0.0)	109	0	(0.0)
Total	0	-		65	0	(0.0)	90	0	(0.0)	155	0	(0.0)
U.S.A.												
Minnesota												
ISP	0	-		28	3	(10.7)	28	6	(21.4)	56	9	(16.1)
CR	0	-		28	1	(3.6)	28	2	(7.1)	56	3	(5.4)
CSP	0	-		28	1	(3.6)	28	4	(14.3)	56	5	(8.9)
Total	0	-		84	5	(6.0)	84	12	(14.3)	168	17	(10.1)
Rhode Island												
TPSK	50	9	(18.0)	0	-		0	-		50	9	(18.0)
HISK	50	3	(6.0)	0	-		0	-		50	3	(6.0)
Total	100	12	(12.0)	0	-		0	-		100	12	(12.0)

* Established populations: PVPP = Pembina Valley Provincial Park; ST = Stanley Trail; ISP = Itasca State Park; CR = Camp Ripley; CSP = St. Croix State Park; PPNP = Point Pelee National Park; LPPP = Long Point Provincial Park; TPSK = Trustom Pond, South Kingstown; HISK = Hazard Island, South Kingstown.
n = no. of individual ticks examined.

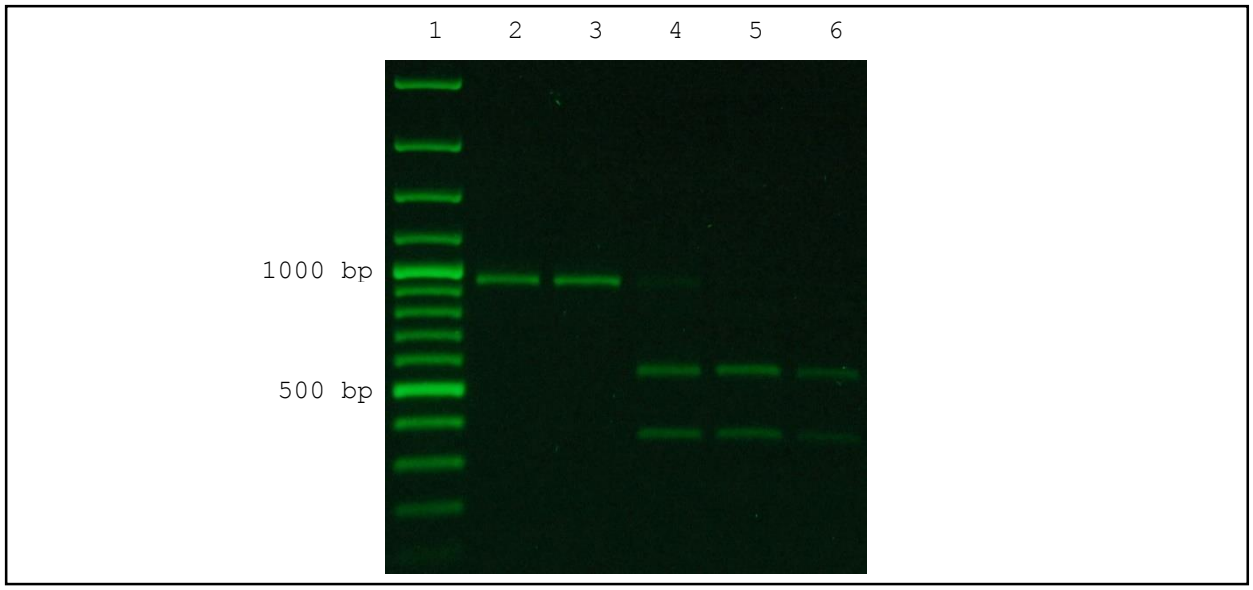


Figure 8.2. Agarose gel showing the three different RFLP patterns of the 16S rDNA of *A. phagocytophilum*. Lane 1 contains a standard DNA ladder (GeneRuler 100 bp Plus DNA Ladder 100 to 3000 bp, Thermo Fisher Scientific). Lanes 2 and 3 contain the undigested amplicons (~920 bp) of two *A. phagocytophilum*-containing samples of ticks that were collected from Itasca State Park (ISP), Minnesota, U.S.A. The RFLP pattern for the Ap-ha strain is represented in these lanes. Lanes 5 and 6 contain the digested amplicons (~360 and ~560 bp) of two *A. phagocytophilum*-containing samples of ticks that were collected from Camp Ripley State Park and St. Croix State Park, Minnesota, U.S.A., respectively. The RFLP pattern for the Ap-variant 1 strain is represented in these lanes. In lane 4, there is a mixture of both undigested and digested amplicons of an *A. phagocytophilum*-containing sample of a tick that was collected from ISP. This third RFLP pattern is consistent with that expected for a sample containing a mixture of both the Ap-ha and Ap-variant 1 strains.

screened using PCR coupled with SNP and/or RFLP and/or DNA sequence analyses, while the Ap-variant 1 strain was found in 42.6% of the PCR-positive ticks (Table 8.3). A single tick contained a mixture of both strains.

Given the small samples sizes for some localities, comparisons of the proportions of *I. scapularis* individuals infected with the Ap-ha relative to the Ap-variant 1 strain were only made on a large spatial scale (Table 8.3). In the "western" region, including Alberta and Manitoba, Canada, as well as Minnesota, U.S.A., the proportion of ticks infected with the Ap-ha strain of *A. phagocytophilum* was 80.8%. The proportions of *A. phagocytophilum*-infected ticks carrying the Ap-ha strain of the bacterium in the "central" (i.e., Ontario and Quebec) and "eastern/Atlantic" (i.e., Rhode Island/New Brunswick, Prince Edward Island, Nova Scotia, and Newfoundland) regions were the same at 47.1%. There was no significant difference in the proportion of ticks infected with *A. phagocytophilum* within each of three geographical regions ("western": $\chi^2_2 = 0.35$, $p > 0.05$; "central": $\chi^2_1 = 1.40$, $p > 0.05$; and "eastern/Atlantic": $\chi^2_4 = 0.64$, $p > 0.05$). In contrast, a significantly greater ($\chi^2_2 = 10.18$, $p < 0.01$) proportion of ticks in the "western" region were infected with the Ap-ha strain when compared to ticks in the "central" and "eastern/Atlantic" regions.

8.4.2. Molecular analyses and genetic diversity of the different *ankA* strains of *A. phagocytophilum*

A single band of the expected size (~890 bp) for the ANK-F1/LA1 primer pair was detected on agarose gels upon electrophoresis for amplicons obtained from the total gDNA of 76 of the 94 *A. phagocytophilum*-infected ticks, as determined by PCR of the 16S rRNA and *msp2* genes (Fig. 8.3). Bands were not detected on agarose gels for the negative control (i.e., no gDNA) samples. A single band corresponding to the size of the amplicons expected for primer pairs: ANK-F2/LA1 (~850 bp) and LA6/LA1 (~450 bp), was also detected on agarose gels upon electrophoresis for each of the *A. phagocytophilum*-containing samples that were subjected to the additional PCR assays (not shown). Bands were always absent from the agarose gels in the lanes where the negative (i.e., no gDNA) controls for each PCR assay were loaded (also not shown). There was 100% concordance in the *ankA* sequences obtained for all samples tested using the primer pairs: ANK-F1/LA1, ANK-F2/LA1, and LA6/LA1.

Table 8.3. The number (no.) of *I. scapularis* that were either adventitious or collected from an established population and infected with the Ap-ha and/or Ap-variant 1 strains of the 16S rRNA gene of *A. phagocytophilum*.

Region Province/State† Locality*	16S rRNA gene strain:						
	Ap-ha:		Ap-variant 1:		Ap-ha & Ap-variant 1:		Total:
	No.	(%)	No.	(%)	No.	(%)	No.
Western region							
Alberta							
AD	1	(100.0)	0	(0.0)	0	(0.0)	1
Manitoba							
PVPP	5	(100.0)	0	(0.0)	0	(0.0)	5
AD	2	(66.7)	1	(33.3)	0	(0.0)	3
Minnesota							
ISP	8	(88.9)	0	(0.0)	1	(11.1)	9
CR	1	(33.3)	2	(66.7)	0	(0.0)	3
CSP	4	(80.0)	1	(20.0)	0	(0.0)	5
Total	21	(80.8)	4	(15.4)	1	(3.8)	26
Central region							
Ontario							
AD	4	(33.3)	8	(66.7)	0	(0.0)	12
Quebec							
AD	12	(54.5)	10	(45.5)	0	(0.0)	22
Total	16	(47.1)	18	(52.9)	0	(0.0)	34
Eastern/Atlantic region							
Rhode Island							
TPSK	5	(55.6)	4	(44.4)	0	(0.0)	9
HISK	0	(0.0)	3	(100.0)	0	(0.0)	3
New Brunswick							
AD	3	(50.0)	3	(50.0)	0	(0.0)	6
Prince Edward Island							
AD	1	(33.3)	2	(66.7)	0	(0.0)	3
Nova Scotia							
AD	6	(54.5)	5	(45.5)	0	(0.0)	11
Newfoundland							
AD	1	(50.0)	1	(50.0)	0	(0.0)	2
Total	16	(47.1)	18	(52.9)	0	(0.0)	34

† Provinces in Canada = Alberta, Manitoba, Ontario, Quebec, New Brunswick, Prince Edward Island, Nova Scotia, and Newfoundland. States in the U.S.A. = Minnesota and Rhode Island.
* Established populations: PVPP = Pembina Valley Provincial Park; ISP = Itasca State Park; CR = Camp Ripley; CSP = St. Croix State Park; TPSK = Trustom Pond, South Kingstown; HISK = Hazard Island, South Kingstown.
% = proportion of the Ap-ha, Ap-variant 1, and mix (both Ap-ha & Ap-variant 1) strains expressed as a percentage of the total number of infected ticks.
AD = adventitious.

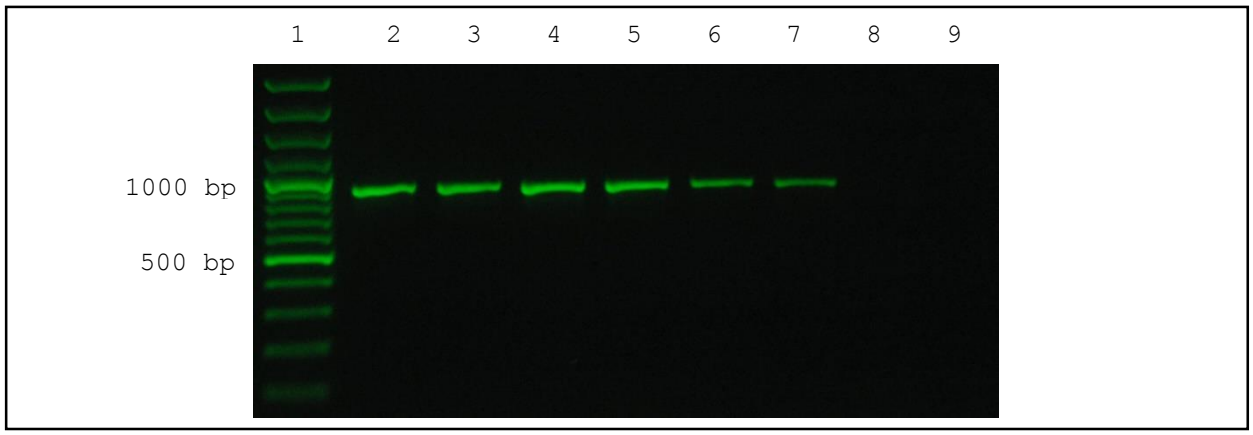


Figure 8.3. Agarose gel showing that the size of the amplicons obtained via PCR of the *ankA* gene of *A*. *phagocytophilum* using the primer pair ANK-F1/LA1 was consistent with that which was expected (~890 bp). Lane 1 contains a standard DNA ladder (GeneRuler 100 bp Plus DNA Ladder 100 to 3000 bp, Thermo Fisher Scientific). Lanes 2-7 contain amplicons of the *ankA* gene of *A*. *phagocytophilum* from samples of ticks collected in Itasca State Park, St. Croix State Park, and Camp Ripley (CR) (Minnesota, U.S.A.), as well as in Pembina Valley Provincial Park (Manitoba, Canada). The amplicon band, although barely visible, of a tick sample collected from CR that was previously determined to be PCR-positive for *A*. *phagocytophilum* by PCR targeting the *ankA* gene is present in lane 8. No amplicons were obtained from the negative (i.e., no gDNA) control (lane 9).

There were 54 variable positions in the 825 bp alignment of the 76 *ankA* sequences representing the adventitious *I. scapularis* and those sampled from the established populations in the present study (Table 8.4). The variable positions were distributed relatively evenly across the length of the alignment. The mutational changes at the variable positions in the alignment consisted of 20 purine transitions, 5 pyrimidine transitions, 23 transversions, 3 indels, and 3 multiple mutational changes. A total of 13 different sequence types (i.e., strains), which differed from one another by 1-43 bp, was detected among the aligned sequences. Of these sequence types, eleven (i.e., CK1, CK3-CK6, and CK8-CK13) were unique to the present study and two (i.e., CK2 and CK7) were detected previously (see Tables 8.1 and 8.4). Interestingly, relative to strains CK7 and CK11, the mutational changes in some strains were heavily concentrated in the first 500 bp of the alignment, while the mutational changes in other strains were almost exclusively found in the last 300 bp of the alignment.

The most common strains detected in the present study were CK7 and CK2, which comprised 22.4% and 21.1% of the *A. phagocytophilum*-infected ticks, respectively (Table 8.5). The next most common strains were CK4 (17.1%), CK6 (14.5%), and CK3 (11.8%). The remaining strains were rare (i.e., represented by one or two infected ticks). A single tick collected from the established population in St. Croix State Park was infected with both strains CK3 and CK4.

Fig. 8.4 shows which *ankA* strains of *A. phagocytophilum* occur in "western" Canada (i.e., Manitoba), "eastern" Canada (i.e., Ontario, Quebec, New Brunswick, Prince Edward Island, Nova Scotia, and Newfoundland), "western" U.S.A. (i.e., Minnesota), and "eastern" U.S.A. (i.e., Rhode Island). In the "eastern" areas of Canada, the number of different strains detected among the 48 samples that were PCR-positive for *A. phagocytophilum* was 13. In the "western" areas of Canada, the number of different *ankA* strains detected among the five PCR-positive samples was one. In the "western" and "eastern" regions of the U.S.A., the number of different *ankA* strains was nearly equal (i.e., four strains were detected among 14 PCR-positive samples and three strains were detected among nine PCR-positive samples, respectively).

The most prevalent strain of *A. phagocytophilum* in infected ticks collected from the "western" region of Canada (i.e., Manitoba) and the U.S.A. (i.e., Minnesota) was CK2 (78.9%) (Table 8.5). Strains CK5-CK13 were not detected in this geographical area. The most frequently detected strain in the "central" region (i.e., Ontario and Quebec) was CK7 (34.5%). Strains

Table 8.4. The variable positions in the aligned *ankA* sequences of *A. phagocytophilum* that were detected in the present study (CK1-CK13) or obtained from GenBank. A dot (.) indicates the same nucleotide as in the sequence of CK7, a dash (-) represents a deletion, and a tilde (~) denotes a missing nucleotide. The sequences comprised three clades (numbered boxes).

Strain/Strain representative[a]		Alignment position:
		1 1 1 2 2 2 3 3 3 3 3 4 4 4 4 4 4 4 4 4 4 4 5 5 5 5 5 5 6 6 6 6 6 6 6 6 6 6 6 7 7 7 7 7 7 7 8 8
		1 2 2 2 2 3 4 8 8 0 1 9 1 5 7 0 4 5 5 9 0 0 0 1 2 5 5 6 6 9 9 0 0 4 4 4 5 0 1 1 1 1 2 2 2 3 4 4 5 1 1 2 6 7 9 9 9 1 2
		6 2 3 5 6 4 9 5 8 3 8 4 5 1 3 0 8 0 9 2 3 4 5 0 5 2 8 0 2 3 9 0 5 7 8 9 3 4 3 5 6 7 9 0 3 8 8 4 5 3 2 8 9 5 6 0 1 6 1 3
CK7[b]	3	A G G G T G A A T A A C C A T A T G T A A G G G T T G A G C T G G A A T A A T T T T G G A C C A A G C G A A A A C C A ~
CK11		. . . . . G . . . . .
CK13		C . A A . A . . . . . T . A . . . . . C . . G G . - - - A A G . A T . . A . G . M G A T G .
CK3		C . A A . A . . . . . T . A . . . . . C . . G G . - - - A A . . A T . . A . G . C G A T G .
CK5		~ . . . . . C . . G G . - - - A A . . A T . . A . G . C G A T G .
CK9	1	. . . . . C . . G G . - - - A A . . G T . . A . G . C G A T G .
CK12		C A A . . A . . . . . C . . G G . - - - A A . A G T . . A . G . C G A T G .
CK4		C A A . . A . . . . . C . . G G . - - - A A . A G T . . A . G . . G A T G .
CK8		C A A . . A . . . . . G . . . . . C . . G G . - - - A A . A G T . . A . G . . G A T G .
CK10		C A A . . A . . . . . A . . . . . C . . G G . - - - A A . A G T . . A . G . . G A T G .
DQ320650_MN-29-1[c]		~ ~ ~ ~ ~ . . . . . A . . . . . C . . G G . - - - A A . A ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ .
DQ320652_MN-61-2		~ ~ ~ ~ ~ . . . . . A . . . A . . . . . C . . G G . - - - A A . A ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ .
DQ320648_RI-1[d]		~ ~ ~ ~ ~ . . . . . A . . . . . C . . G G . - - - A A . . ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ .
DQ320653_MN-38		~ ~ ~ ~ ~ A . . . . . C . . G G C - - - A C . A ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ .
DQ320654_MN-53-1		~ ~ ~ ~ ~ A . . . . . T . A . . . . . C . . G G . - - - A . . . ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ .
CK1		C . C . C A G T G G G T T G G T G A G . T A T A . . A T A G C T C . . G . . . - - - . . . . .
CK2[e]	2	C . C . C A . T G G G . T G G T G A G . T A T A . . A T A G C T C . . G . . . - - - . . . . . T . . . . .
CK6		C . C . C A . T G G G . T G G T G A G . T A T A . . A T A G C T C . . . . G . - - - . . . . .
AF100890_WI1		C . C . C A . T G G G . T G G T G A G . T A T A . . A T A G C T C . . G . . . - - - . . . . . G T . T . . . . . A
AF356512_WI1		C . C . C A . T G G G . T G G T G A G . T A T A . . A T A G C T C . . G . . . - - - . . . . . T . C . . . . . A
AF100882_EE		C . C . C A . T G G G . . G G T G A G . T A T A . . A T A G C T C . . G . . . - - - . . . . . G . . . A
AF153716_strMRK		C . C . C A . T G G G . . G G T G A G . T A T A . . A T A G C T C . . G . . . - - - . . . . . G . . . T

[a] Strains CK1-CK4, CK6-CK8, and CK10-CK12 were based on sequences of only 819 bp (i.e., missing 6 bp at the 3′ end). Strains CK5, CK9, and CK13 were based on sequences of only 801 bp, 812 bp, and 807 bp, respectively (i.e., missing 18 bp, 7 bp, and 12 bp, respectively, at the 5′ end and 6 bp each at the 3′ end). Sequences corresponding to GenBank accession nos. beginning with DQ were based on only 607 bp (i.e., missing 27 bp at the 5′ end and 191 bp at the 3′ end), except for DQ680013, which was based on only 610 bp (i.e., missing 215 bp at the 5′ end).

[b] Also includes AF100883_NY1, AF100884_NY2, AF100885_NY3, CP000235_strHZ, GU236808_96HE58, AF020521_strUSG3, and DQ680013.

[c] Also includes DQ320651_MN-62-6.

[d] Also includes DQ320649_RI-2.

[e] Also includes AF100891_WI2, AF100892_WI3, AF100893_WI4, AF047897_strBDS, GU236806_03HE, GU236807_96HE54, and AF100894_CGEagent.

Table 8.5. The number (no.) of *I. scapularis* that were either adventitious or collected from an established population and were infected with a given *ankA* strain of *A. phagocytophilum*.

Region Province/State† Locality*	*ankA* gene strain:													
	CK1:	CK2:	CK3:	CK4:	CK5:	CK6:	CK7:	CK8:	CK9:	CK10:	CK11:	CK12:	CK13:	Total:
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No.
Western region														
Manitoba														
PVPP	0 (0.0)	5 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	5
Minnesota														
ISP	1 (14.3)	6 (85.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	7
CR	0 (0.0)	1 (33.3)	1 (33.3)	1 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3
CSP	0 (0.0)	3 (75.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4‡
Total	1 (5.3)	15 (78.9)	1 (5.3)	1 (5.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	19‡
Central region														
Ontario														
AD	1 (10.0)	1 (10.0)	1 (10.0)	3 (30.0)	0 (0.0)	0 (0.0)	1 (10.0)	1 (10.0)	0 (0.0)	1 (10.0)	0 (0.0)	1 (10.0)	0 (0.0)	10
Quebec														
AD	0 (0.0)	0 (0.0)	3 (15.8)	4 (21.1)	0 (0.0)	2 (10.5)	9 (47.4)	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.3)	0 (0.0)	0 (0.0)	19
Total	1 (3.4)	1 (3.4)	4 (13.8)	7 (24.1)	0 (0.0)	2 (6.9)	10 (34.5)	1 (3.4)	0 (0.0)	1 (3.4)	1 (3.4)	1 (3.4)	0 (0.0)	29

Table 8.5. Continued.

Region Province/State† Locality*	*ankA* gene strain:													
	CK1:	CK2:	CK3:	CK4:	CK5:	CK6:	CK7:	CK8:	CK9:	CK10:	CK11:	CK12:	CK13:	Total:
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	No.
Eastern/Atlantic region														
Rhode Island														
TPSK	0 (0.0)	0 (0.0)	0 (0.0)	2 (28.6)	0 (0.0)	5 (71.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	7
HISK	0 (0.0)	0 (0.0)	1 (50.0)	1 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2
New Brunswick														
AD	0 (0.0)	0 (0.0)	0 (0.0)	1 (20.0)	1 (20.0)	0 (0.0)	3 (60.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	5
Prince Edward Island														
AD	0 (0.0)	0 (0.0)	1 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	1 (33.3)	0 (0.0)	1 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3
Nova Scotia														
AD	0 (0.0)	0 (0.0)	2 (20.0)	1 (10.0)	0 (0.0)	3 (30.0)	3 (30.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (10.0)	10
Newfoundland														
AD	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1
Total	0 (0.0)	0 (0.0)	4 (14.3)	5 (17.9)	1 (3.6)	9 (32.1)	7 (25.0)	0 (0.0)	1 (3.6)	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.6)	28

† Provinces in Canada = Alberta, Manitoba, Ontario, Quebec, New Brunswick, Prince Edward Island, Nova Scotia, and Newfoundland. States in the U.S.A. = Minnesota and Rhode Island.
* Established populations: PVPP = Pembina Valley Provincial Park; ISP = Itasca State Park; CR = Camp Ripley; CSP = St. Croix State Park; TPSK = Trustom Pond, South Kingstown; HISK = Hazard Island, South Kingstown.
% = proportion of the different *ankA* strains expressed as a percentage of the total number of infected ticks.
‡ a female tick collected from CSP was infected with strains CK3 and CK4, and is accounted for in the totals, as well as in the proportions of the different strains.
AD = adventitious ticks only.

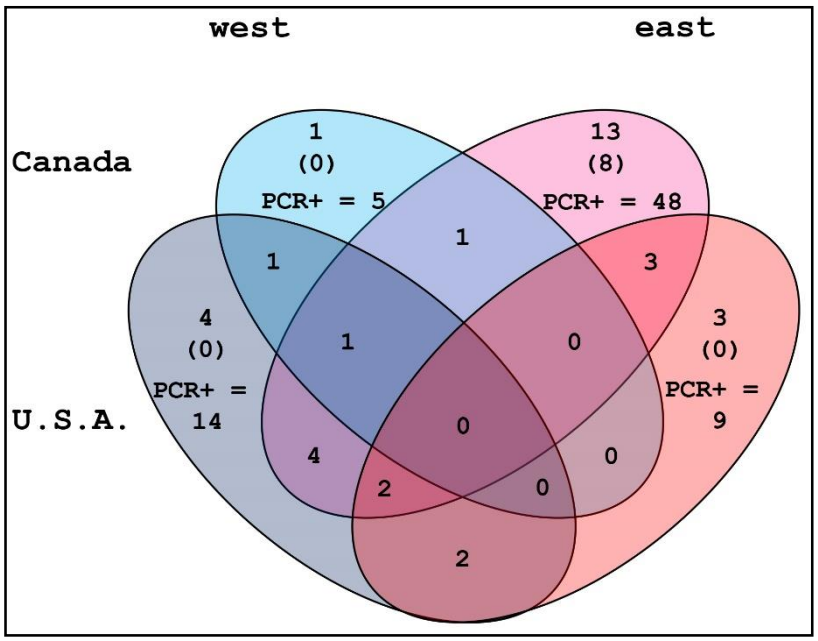


Figure 8.4. Venn diagram representing the number of *ankA* strains of *A*. *phagocytophilum* found in and shared among key geographical areas. Ovals correspond to: "western" Canada (light blue), "eastern" Canada (pink), "western" U.S.A. (dark blue), and "eastern" U.S.A. (red). The number of strains that were found within a particular geographical region only (in parentheses), as well as the number of *I*. *scapularis* that were PCR-positive (PCR+) for *A*. *phagocytophilum* when targeting the *ankA* gene in each region are also reported. Strains of *A*. *phagocytophilum* in PCR-positive *I*. *scapularis* collected from established populations (n = 28) and those of the adventitious ticks (n = 48) have been included.

CK4 (24.1%) and CK3 (13.8%) were also prevalent in this geographical region. Strains CK1, CK2, CK8, and CK10-CK12 were each represented by a single *A. phagocytophilum*-infected tick, and strains CK5, CK9, and CK13 were not detected in this geographical region. Of the *A. phagocytophilum*-infected *I. scapularis* collected from the "eastern/Atlantic" regions (i.e., Rhode Island/New Brunswick, Prince Edward Island, Nova Scotia, and Newfoundland), the most prevalent strain of *ankA* was CK6 (32.1%), followed by CK7 (25.0%), CK4 (17.9%), and CK3 (14.3%). Three strains (i.e., CK5, CK9, and CK13) were each represented by a single tick. Strains CK1, CK2, CK8, and CK10-CK12 were not detected in this geographical area.

Fig. 8.4 also shows that the number of *ankA* strains that were shared between pairs of the four geographical areas varied from zero (between "western" Canada and "eastern" U.S.A.) to four (between "western" U.S.A. and "eastern" Canada). Notably, the single strain that was found in "western" Canada was also detected in the "western" U.S.A. Similarly, all three of the *ankA* strains detected in the "eastern" U.S.A. were also detected in "eastern" Canada. The single strain that was found in "western" Canada was also found in "eastern" Canada; however, only two of the three strains detected in the "eastern" U.S.A. were also found in the "western" U.S.A. At least three times as many *ankA* strains were detected in the "eastern" areas of Canada and U.S.A., as compared to the "western" areas (i.e., thirteen strains, as compared to four). Comparison of the *ankA* strains detected in Canada with those detected in the U.S.A. revealed that the number of *ankA* strains in Canada was at least two-and-a-half times the number of strains in the U.S.A. (i.e., thirteen strains, as compared to five). However, the number of strains in common between the "western" and "eastern" geographical areas of the U.S.A. (i.e., two strains) was greater than that number in common between the "western" and "eastern" areas of Canada (i.e., a single strain). No single *ankA* strain of *A. phagocytophilum* was common to all four geographical areas.

8.4.3. Phylogeographical and phylogenetic analyses

Fig. 8.5 shows the relationships among *ankA* strains of *A. phagocytophilum* that have been detected in North America in this (i.e., CK1-CK13) and other (Caturegli et al. 2000, Massung et al. 2000) studies. There was 100% support for two clades in the phylogenetic tree, based on the NJ analyses. These clades (i.e., Clades 1 and 3) were comprised of strains CK3-CK5, CK8-CK10, CK12, and CK13, as well as CK7 and CK11 (respectively). A third clade

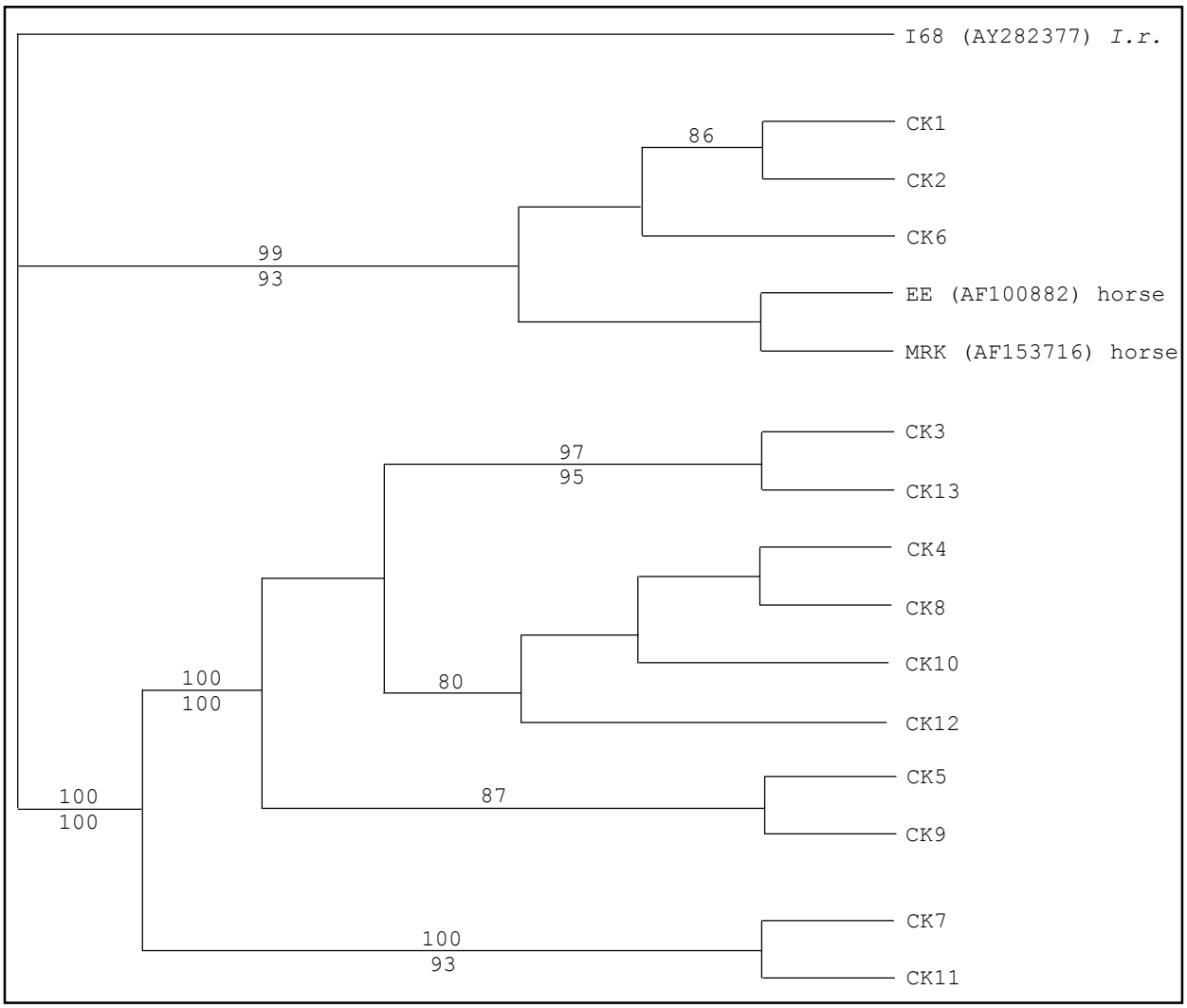


Figure 8.5. Phylogenetic tree constructed using the neighbour-joining (NJ) and maximum parsimony (MP) methods (bootstrap replicates = 1000 and 100, respectively) in PAUP (Swofford 2002) showing the relationships among the *ankA* strains (aligned over 825 bp) of *A. phagocytophilum* detected in different geographical areas of North America and reported in this (strains CK1-CK13) and other studies. Bootstrap support (≥75%) for the different groups is indicated above (NJ) and below (MP) the pertinent branches of the tree. Also included (in round brackets) are the GenBank accession numbers for the EE and MRK strains, which were isolated from horses (Caturegli et al. 2000, Massung et al. 2000). An arbitrary sequence (GenBank accession number in round brackets) obtained from an *I. ricinus* individual collected in Germany was used as the outgroup (von Loewenich et al. 2003).

(i.e., Clade 2), which was comprised of strains CK1, CK2, CK6, EE, and MRK, had 99% support based on the NJ method. Bootstrap support for these three clades based on the MP analyses was similar to that of the NJ method. There was also very strong statistical support (i.e., 97% based on NJ and 95% based on MP) for a fourth clade (i.e., CK3 and CK13), although it belonged to one of the three main clades. All *A. phagocytophilum* defined as the Ap-variant 1 strain based on analyses of the 16S rRNA gene belonged to Clade 1 based on analyses of *ankA*. Similarly, all *A. phagocytophilum* defined as the Ap-ha strain based on analyses of the 16S rRNA gene belonged to Clades 2 and 3.

A second phylogenetic tree (Fig. 8.6), which was based on the amino acid sequences of the different *ankA* strains and constructed using the NJ method, also shows very strong boostrap support for three main clades: Clade 1, which was comprised of strains CK3-CK5, CK8-CK10, CK12, and CK13; Clade 2, which included strains CK1, CK2, CK6, EE, and MRK; and, Clade 3, which was comprised of strains CK7 and CK11. As in Fig. 8.5, strains CK3 and CK13 formed a group to the exclusion of the other members of Clade 1.

Fig. 8.7A depicts the relationships among 40 strains of *A. phagocytophilum* representing 136 DNA sequences aligned over 832 bp detected in North America and Europe in this study, others, and/or directly deposited in GenBank (see Tables 8.1 and 8.4). The 22 strains detected in North America differed from the 18 strains detected in Europe by at least 21 bp. Among the strains detected in North America (Fig. 8.7B), the number of differences between any two strains in the minimum spanning network ranged from 1-43 bp. There were three distinct clades of *ankA* strains in North America, which corresponded to the three main clades with strong bootstrap support that were detected via the phylogenetic analyses. Clade 1 consisted of 13 strains, which differed from one another by at most 8 bp. Clade 2 was comprised of seven strains, differing from one another by a maximum of 5 bp. The two strains in Clade 3 differed from one another by a single nucleotide. The *ankA* strains comprising Clade 1 differed from those comprising Clade 2 by a minimum of 25 base pairs, while Clades 1 and 3 differed by at least 9 bp.

The number of fixed differences in DNA sequence between Clades 1 and 2 was 32, whereas the number of fixed differences between Clades 1 and 3, and between 2 and 3, were 15 and 28, respectively (Table 8.4). The Ap-ha group of *ankA* strains (i.e., Clades 1 and 3) did not represent a monophyletic assemblage, because Clade 3 was genetically more similar to Clade 1 than to Clade 2. The majority of the mutational changes in the DNA sequences corresponded to

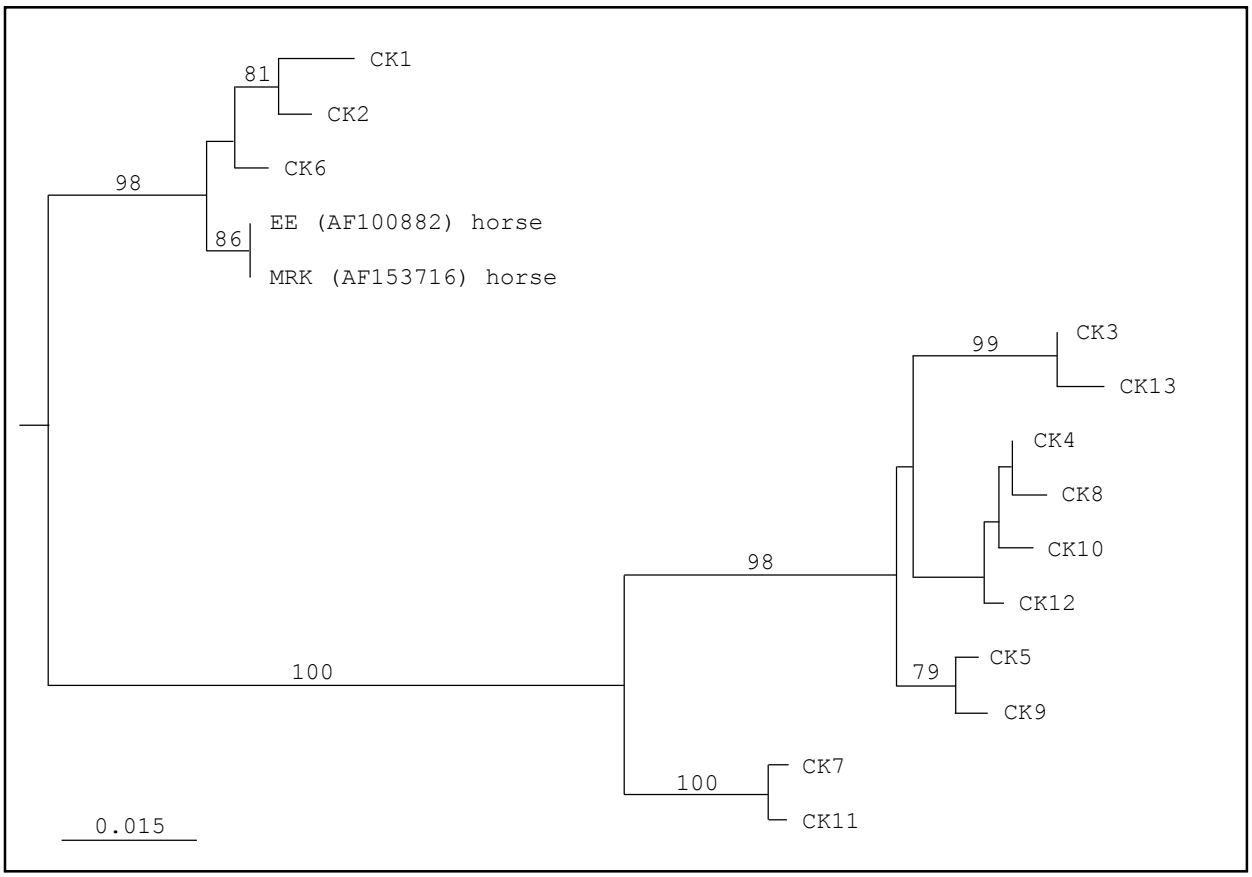


Figure 8.6. Unrooted phylogenetic tree constructed using the neighbour-joining method (bootstrap replicates = 1000) in PAUP (Swofford 2002) showing the relationships among the amino acid sequences corresponding to the *ankA* strains (aligned over 825 bp) of *A. phagocytophilum* detected in different geographical areas of North America and reported in this (strains CK1-CK13) and other studies. The scale bar indicates the number of inferred substitutions per variable site. Bootstrap support (≥75%) for the different groups is indicated above the pertinent branches of the tree. Also included (in round brackets) are the GenBank accession numbers for the DNA sequences of the EE and MRK strains, which were isolated from horses (Caturegli et al. 2000, Massung et al. 2000).

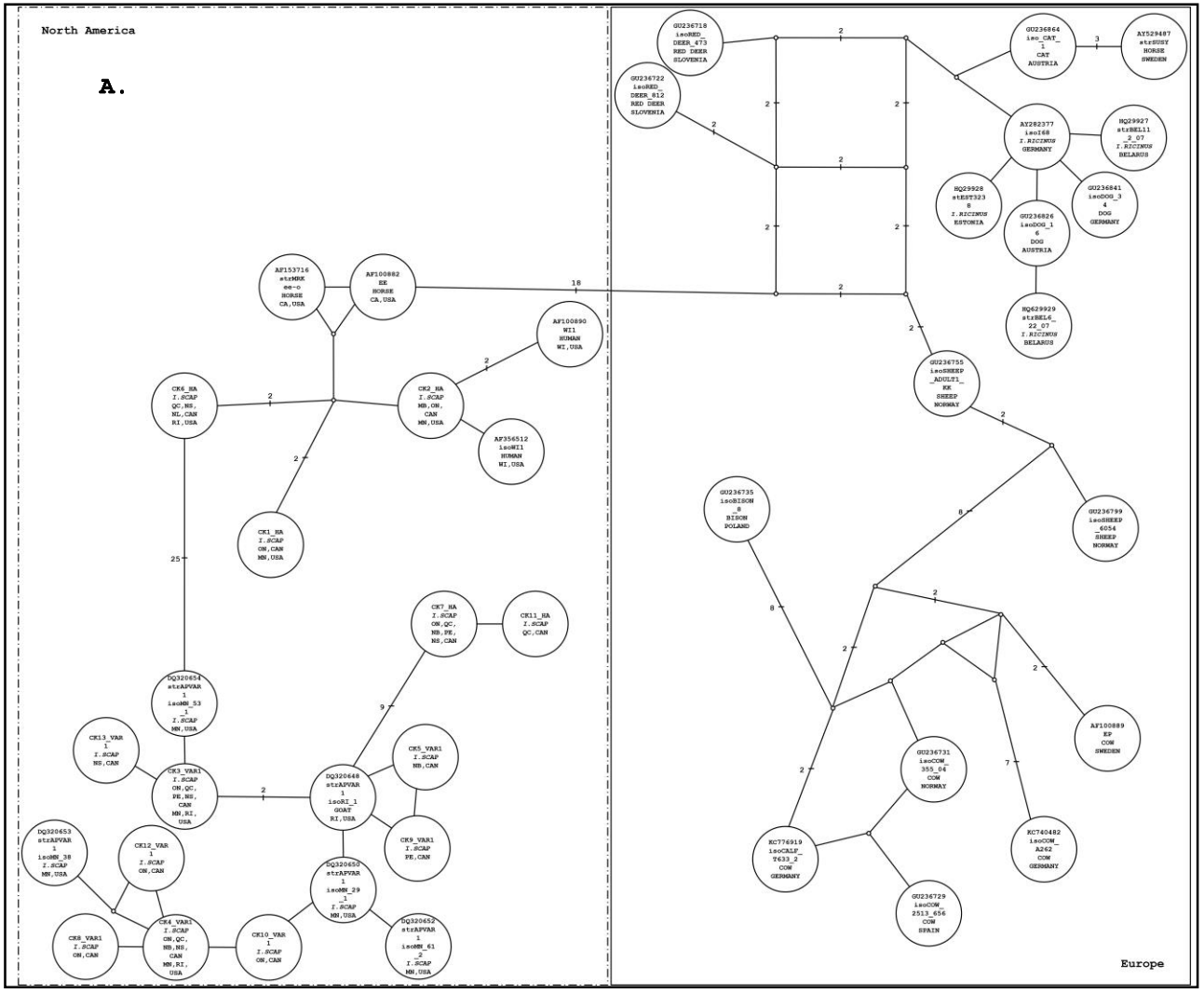


Figure 8.7. A minimum spanning network depicting the relationships among *ankA* strains of *A. phagocytophilum* reported in this (strains CK1-CK13) and other studies (Storey et al. 1998, Pusterla et al. 1999, Caturegli et al. 2000, Massung et al. 2000, Lodes et al. 2001, Von Loewenich et al. 2003b, Dunning Hotopp et al. 2006, Massung et al. 2007, Domingos et al. 2011, Scharf et al. 2011, Katargina et al. 2012, Henniger et al. 2013) and/or that were directly deposited in GenBank (see Table 8.1). The number of nucleotide differences between pairs of DNA sequences (circles) is one unless otherwise indicated. Putative haplotypes are represented by small open circles. A) European strains are contained within the rectangle on the right (outlined with solid black), whereas North American strains are confined within the rectangle on the left (outlined with long-dash-dot pattern). B) North American strains only.

Figure 8.7. Continued. Strains detected in North America are denoted by yellow- (California), blue- ("western"), grey- ("western" and "eastern"), and red- ("eastern") filled circles. Clades 1-3 are contained within the dashed rectangles.

changes in the composition of the amino acid sequences (Table 8.6). The number of fixed differences in the amino acid sequences among clades ranged from 11 to 28. The positions of the variable amino acids in some strains relative to CK7 and CK11 in the alignment were skewed towards the N- or C-terminal ends of the alignment. For example, all 11 of the fixed amino acid differences between Clades 1 and 3 were found near the C-terminal end (i.e., within the last ~100 aa). Similarly, all but one of the fixed amino acid differences between Clades 2 and 3 occurred within the first ~170 aa of the aligned sequences.

8.5. Discussion

The primary objective of the present chapter was to examine the genetic variability in the *ankA* gene of *A. phagocytophilum* infecting *I. scapularis*. However, nPCR analyses targeting the 16S rRNA gene were also used to determine the prevalence of *A. phagocytophilum* infection in *I. scapularis* collected from nine established populations. Due to small sample sizes for some localities, comparisons of the proportions of *I. scapularis* individuals infected with the Ap-ha or Ap-variant 1 strain of *A. phagocytophilum* were only made on a large spatial scale. There were no significant differences in the proportion of ticks infected within each of the three geographical regions considered (i.e., the "western," "central," and "eastern/Atlantic" regions). However, a significantly greater proportion of *A. phagocytophilum*-infected ticks in the "western" region contained the Ap-ha strain, as compared to those in the "central" and "eastern/Atlantic" regions. This suggests that the potential risk for human exposure to the Ap-ha strain may differ among geographical regions in Canada, as well as between the Midwest and Northeast regions of the U.S.A.; however, additional sampling is required to test this hypothesis. The findings from the Canadian data were consistent with those reported in Chapter 7, which were based on different samples. Unfortunately, it was not possible to compare my findings with those of studies conducted in the U.S.A. for several reasons. Firstly, sample sizes were too small in some of these other studies (e.g., two of four samples that were PCR-positive for the bacterium were sequenced; Layfield and Guilfoile 2002). Secondly, there is homogeneity in the proportions of ticks infected with the Ap-ha strain relative to the Ap-variant 1 strain within and among localities covering a broad geographical area (i.e., two states in the Midwest and two in the Northeast) (Steiner et al. 2008). Thirdly, the extent of the variability in the proportion of ticks infected with

Table 8.6. The variable positions in the aligned amino acid sequences of AnkA of *A. phagocytophilum* that were detected in the present study (CK1-CK13), as compared to others (Caturegli et al. 2000, Massung et al. 2000). A dot (.) at an alignment position indicates the same amino acid as in the sequence of CK7, a dash (-) represents a deletion, and a tilde (~) denotes an undetermined amino acid. Amino acids have been coloured according to the biochemical properties of their side chains: red = acidic, blue = basic, yellow = polar, and green = non-polar. The sequences comprised three clades (numbered boxes).

| Strain/ Strain representative | | Alignment position: | | |

the Ap-ha strain, as compared to the Ap-variant 1 strain, within a single state (e.g., Rhode Island; Massung et al. 2002, and Pennsylvania; Courtney et al. 2003) was equivalent to that detected in Minnesota in the Midwest when compared to Rhode Island in the Northeast (present study). Lastly, there is evidence to suggest that the proportion of ticks infected with the Ap-variant 1 strain, as compared to the Ap-ha strain, may be biased depending on the host from which they were collected (e.g., white-tailed deer are not known to harbour the Ap-ha strain; Massung et al. 2005).

A large proportion of the samples that were PCR-positive for *A. phagocytophilum* based on the 16S rRNA gene were further characterized based on DNA sequences of *ankA*. A total of 13 strains (i.e., 13 different *ankA* sequence types) were detected. This number of *ankA* sequence variants of *A. phagocytophilum* was considerably greater than that previously reported for North America (Massung et al. 2000, Shukla et al. 2007). However, the study by Massung et al. (2000) primarily examined genetic variants of *A. phagocytophilum* infecting humans in New York State and Wisconsin, while the study by Shukla et al. (2007) only examined genetic variants of *A. phagocytophilum* infecting dogs in Washington State.

The phylogenetic analyses conducted on both the DNA sequence data and the amino acid sequence data showed separation of the different *ankA* variants of *A. phagocytophilum* into three distinct clades. There was strong bootstrap support for each of these clades. In addition, there were distinct associations between *ankA* strains and either the Ap-ha or Ap-variant strains of the 16S rRNA gene. Five *ankA* strains (i.e., CK1, CK2, CK6, CK7, and CK11) were all defined as the Ap-ha strain based on the 16S rRNA gene, whereas the other *ankA* strains (i.e., CK3-CK5, CK8-CK10, CK12, and CK13) were defined as the Ap-variant 1 strain of the 16S rRNA gene. These findings (i.e., of associations between *ankA* strains and either the Ap-ha or Ap-variant 1 strains of the 16S rRNA gene) are consistent with those of Shukla et al. (2007). In their study, variants 1, 2, 4, and 5 of the 16S rRNA gene were associated with one set of *ankA* strains, while variant 3 was associated with another set of *ankA* strains. All of the *ankA* strains defined as the Ap-variant 1 strain in the present study formed a monophyletic group (i.e., Clade 1). In contrast, the *ankA* strains defined as Ap-ha strain did not form a monophyletic assemblage, but belonged to one of two different groups (i.e., Clades 2 and 3). Interestingly, Clade 3 (corresponding to the Ap-ha strain) is genetically more similar to Clade 1 (corresponding to the Ap-variant 1 strain) than to Clade 2 (also corresponding to the Ap-ha strain).

It is known that AnkA, an apparently unique protein of *A. phagocytophilum*, binds to granulocyte DNA and nuclear proteins in the host and causes alterations to host histones, chromatin structure, and gene expression (Caturegli et al. 2000, Park et al. 2004, Thomas et al. 2005, Garcia-Garcia et al. 2009, Rennoll-Bankert and Dumler 2012). Specifically, AnkA interacts with AT-rich regions in the promoter of the *CYBB* gene, which codes for $gp91^{phox}$ (i.e., a component of NADPH oxidase), in human cells (Garcia-Garcia et al. 2009, Rennoll-Bankert and Dumler 2012). Therefore, the major differences in the distribution of the alterations in nucleotide and amino acid sequences among *ankA* clades may correspond to structural and functional differences in AnkA.

The amino acid sequences of AnkA for the two clades of Ap-ha strains (i.e., Clades 2 and 3) differ from one another, primarily, over the first part of the sequence alignment. Approximately half of these variable positions represent relaxed conserved residues at which the biochemical properties of the site are preserved despite substitutions (Studer et al. 2013). In contrast, the amino acid sequences of AnkA for the clade of Ap-variant 1 strains (i.e., Clade 1), when compared to the clades of Ap-ha strains (i.e., Clades 2 and 3), largely differ from one another in the second part of the amino acid sequence. This may correspond to differences in evolutionary rate and/or physicochemical properties between *A. phagocytophilum* of Clade 1 and those of Clades 2 and 3 (Studer et al. 2013).

Given that AnkA of *A. phagocytophilum* interacts with host granulocyte DNA and nuclear proteins (Park et al. 2004, Garcia-Garcia et al. 2009) and that the orthologous DNA and proteins with which AnkA interacts likely differ in sequence depending on the host, a possible hypothesis is that the fixed differences in the latter parts of these sequence alignments may be related to distinct host associations of the strains. Some evidence supporting this hypothesis has already been found in Europe (Scharf et al. 2011), as well as in North America (Massung et al. 2002, Massung et al. 2003b, Foley et al. 2008b, Reichard et al. 2009). For example, in Europe, it has been shown that *ankA* clusters correlate with distinct host species, and it was suggested that particular mutational changes in a region at the C-terminal end of AnkA sequences may be related to biological differences among the clusters (Scharf et al. 2011). In North America, it has been shown that the Ap-variant 1 strain of *A. phagocytophilum* (based on the 16S rRNA gene) is carried by *I. scapularis*, but does not readily infect either white-footed (*Peromyscus leucopus*) or DBA/2 (*Mus musculus*) mice (Massung et al. 2003b). Likewise, associations between certain

16S rRNA gene strains of *A. phagocytophilum* and its hosts have been discovered in Rhode Island, Connecticut, Maryland, and Wisconsin (Belongia et al. 1997, Massung et al. 1998, Massung et al. 2002). For example, although three strains (Ap-variant 1, Ap-ha, and Ap-variant 2) have been detected in *I. scapularis*, only two of these (Ap-ha and Ap-variant 2) have been found in *P. leucopus* (Massung et al. 2002). Three strains of *A. phagocytophilum* (Ap-ha, Ap-variant 3, and Ap-variant 4) have been detected in the eastern chipmunk (*Tamias striatus*) (Massung et al. 2002), while only the Ap-variant 1 strain has been detected in white-tailed deer (*Odocoileus virginianus*) (Belongia et al. 1997, Massung et al. 1998). Since it has been shown in the present study, as well as in the study by Shukla et al. (2007), that strains of *A. phagocytophilum* based on sequences of the *ankA* gene correlate with particular strains of the 16S rRNA gene, studies uncovering associations between particular 16S rRNA gene strains of *A. phagocytophilum* and its hosts in North America (Belongia et al. 1997, Massung et al. 1998, Massung et al. 2002, Massung et al. 2003b) provide indirect support for the hypothesis that the unequal distribution of the alterations in the DNA and amino acid sequences among *ankA* clades is associated with distinct host species. Additional evidence has been obtained in the present study to support this hypothesis. For example, Clade 1 comprised *ankA* strains isolated from goats (i.e., vertebrate hosts of *A. phagocytophilum*) and *I. scapularis* (i.e., vector of the bacterium), while Clades 2 and 3 comprised strains isolated from horses, dogs, cats, and humans (i.e., vertebrate hosts of the bacterium) and *I. scapularis*. Further study of the host-pathogen dynamics of *A. phagocytophilum* is required to test the hypothesis that fixed differences in particular regions of *ankA* sequences are related to distinct host associations of the *ankA* clades.

Phylogeographical analysis of the sequence data showed that Clades 1 and 2 consisted of *ankA* strains reported from both "western" and "eastern" regions of North America, whereas Clade 3 contained strains detected in only the "eastern" geographical region. This suggests that, although Clades 1 and 2 display no phylogeographical structure, there may still be sufficient phylogeographical signal in the *ankA* gene of *A. phagocytophilum* in *I. scapularis* to infer, to some degree, the evolutionary history of the species. For example, although Clades 1 and 2 (and 3) probably share a recent common ancestor, the absence of *ankA* strains belonging to Clade 3 in the "western" geographical region, and the frequent detection of strain CK7 in the "eastern" geographical region, suggests that differing selective pressures or dispersal rates may be acting upon *A. phagocytophilum* in the "eastern" region, as compared to the "western" region. This is

consistent with the findings of the phylogeographical study of Humphrey et al. (2010) on *Borrelia burgdorferi*, another *I. scapularis*-borne pathogen. The authors inferred that populations of *B. burgdorferi* in the Midwest and Northeast, U.S.A. were derived from a single pool of highly variable strains, but now suffer a significant barrier(s) to gene flow between these regions (Humphrey et al. 2010). This regional differentiation is thought to have been influenced by a single group of *B. burgdorferi* strains whose members were prevalent in the Northeast, as compared to the Midwest, that have either been prevented from establishing in the Midwest, due to selective pressures, or unable to establish there, due to lower dispersal rates (Humphrey et al. 2010). These findings were consistent with those of Hoen et al. (2009) in that they supported the hypothesis that *B. burgdorferi* populations in the Midwest, U.S.A. originated from populations in the Northeast, U.S.A.

Prior investigations (i.e., Chapters 2, 3, and 6) of the genetic variability in several mitochondrial gene regions of the same *I. scapularis* that were screened for *A. phagocytophilum* in the present study, or a subset thereof, revealed that there was extensive genetic variation in each molecular marker and that there were significant differences in the genetic structure among populations of this tick species. Associations between some sequence types (i.e., haplotypes) of *I. scapularis* and particular geographical areas in Canada were also uncovered. The findings of these studies provide support for the hypothesis that there are different geographical origins in the U.S.A. for blacklegged ticks in different geographical areas of Canada. Since *I. scapularis* is a key vector of *A. phagocytophilum* in North America (Hodzic et al. 1998) and *A. phagocytophilum* infects humans, domestic animals, and wildlife (Bakken et al. 1994, Chen et al. 1994, Massung et al. 2005, Cockwill et al. 2009, Burgess et al. 2012, Johnston et al. 2013), it is of medical and veterinary importance to determine its prevalence in the different geographical areas in which it occurs. Furthermore, some strains of *A. phagocytophilum* are associated with distinct host species. For example, the Ap-ha strain has been isolated from humans exhibiting symptoms of human granulocytic anaplasmosis (Chen et al. 1994, Lovrich et al. 2011), whereas the Ap-variant 1 strain has not been associated with human infection (Massung et al. 1998). Given this, it is of importance to determine the proportion of *I. scapularis* infected with the different 16S rRNA gene strains in North America. This is especially true in Canada, where the distribution of the blacklegged tick has recently expanded and is expected to expand further, due to the transportation of immature (i.e., larval and nymphal) *I. scapularis* via passerines during

spring migration and environmental changes associated with global warming affecting tick reproduction and survival (Klich et al. 1996, Morshed et al. 2005, Ogden et al. 2005, Ogden et al. 2006b, Ogden et al. 2008c, Ogden et al. 2008d, Ogden et al. 2008e, Ogden et al. 2009, Ogden et al. 2013b). Thus, in a recent study (Chapter 7) the prevalence of *A. phagocytophilum* in blacklegged ticks from southern Canada was determined by PCR analyses targeting the 16S rRNA gene of the bacterium. The results showed that the proportion of ticks infected with *A. phagocytophilum* varied significantly among geographical regions. Examination of the strain variation in the 16S rRNA gene in that study revealed two strains, and that a greater proportion of *A. phagocytophilum*-infected ticks from Manitoba contained the Ap-ha strain than infected ticks in provinces east of Manitoba. This not only suggests that human granulocytic anaplasmosis may represent an emerging disease in southern Canada, but that the human exposure to the Ap-ha strain potentially differs among geographical areas. Moreover, this further supports the hypothesis that blacklegged ticks in Canada originated from different geographical areas in the U.S.A.

This study, which examined strain variation in the *ankA* gene of *A. phagocytophilum*, provides insight into the evolutionary history of an important human and animal pathogen in North America. Phylogeographical analysis suggests that *A. phagocytophilum* strains in the "eastern" geographical region gave rise to those in the "western" geographical region. Therefore, the phylogeography of this bacterium of medical and veterinary importance in North America needs to be examined further using *ankA* as a genetic marker.

CHAPTER 9
GENERAL DISCUSSION

9.1. Objectives

The blacklegged tick, *Ixodes scapularis*, is an important vector of several pathogens (e.g., *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, *Babesia microti*, and the Powassan (POW) virus) (McLean 1963, Spielman 1976, Burgdorfer et al. 1982, Hodzic et al. 1998). The distributional range of this tick species has continued to expand in southern Canada, such that the number of established populations is now greater than 40 (Watson and Anderson 1976, Ogden et al. 2009, Koffi et al. 2012). This range expansion has been attributed to the northwards transport of larval and nymphal ticks by passerine birds during their spring migration from the U.S.A. (Klich et al. 1996, Morshed et al. 2005, Ogden et al. 2008c, Scott et al. 2012), the presence of suitable hosts in southern Canada (Barker et al. 1992, Ogden et al. 2006c, Bouchard et al. 2011, Bouchard et al. 2013), and climatic conditions that are becoming more favourable for tick survival and reproduction (Ogden et al. 2005, Ogden et al. 2006b, Ogden et al. 2008a, Ogden et al. 2014b). Hence, there is an increased risk of exposure for Canadians to blacklegged ticks and the pathogens they carry (Barker and Lindsay 2000, Brownstein et al. 2005, Ogden et al. 2014a). Consequently, there is a need to examine basic biological, ecological, and biogeographical questions about *I. scapularis* and its associated microorganisms. Thus, **the aim of my Ph.D. research was to genetically characterize *I. scapularis* individuals and one of the pathogens that it carries (i.e., *A. phagocytophilum*) to infer the geographical origins of the different populations of this tick species in Canada.**

The body of this thesis is comprised of two major sections. The first section is a collection of studies (Chapters 2-6) that examined the extent of the genetic variability in, as well as the population genetics and phylogeography of, *I. scapularis* using different genetic markers. The second section consists of two studies (Chapters 7 and 8) in which the magnitude of the genetic variation in *A. phagocytophilum* was investigated by targeting two vastly different gene regions in the bacterium. Phylogeographical inferences for *A. phagocytophilum* were made in Chapter 8.

9.2. Genetic variation in *I. scapularis*

Initially, an ~400 bp region of Domains IV and V of the mitochondrial (mt) 16S ribosomal (r) RNA gene was used as a genetic marker to investigate the population genetics and phylogeographical patterns of *I. scapularis* (Chapter 2). This genetic target has often been used in studies of the population genetics of *I. scapularis* (Rich et al. 1995, Norris et al. 1996, Qiu et al. 2002, Trout et al. 2009, Humphrey et al. 2010, Krakowetz et al. 2011, Van Zee et al. 2013). Most of the genetic variability among individual ticks was detected in the "hypervariable" region of Domain V, which is consistent with the findings of other population genetic studies on ticks and other invertebrates (e.g., Black IV and Piesman 1994, Misof et al. 2002, Smith and Bond 2003). A total of 52 haplotypes were detected among 582 ticks, which was 2-7 times greater than the number of haplotypes found in previous studies with samples sizes ranging from approximately 30-650 ticks (Rich et al. 1995, Norris et al. 1996, Qiu et al. 2002, Trout et al. 2009, Humphrey et al. 2010, Krakowetz et al. 2011, Van Zee et al. 2013). The number of haplotypes in the established populations ranged from 7-14; however, the statistical analyses revealed that these values represented an underestimate of the haplotype richness in many of these populations. The existence of these undetected haplotypes could be confirmed by additional sampling from these populations, which would also improve our understanding of the dispersal patterns of *I. scapularis*. The results of the statistical analyses also showed that there were significant differences in the genetic diversity among established populations of blacklegged ticks. Most of this genetic variation (94%) occurred within populations, although significant genetic structuring was found among populations both within and among geographical regions. There was also a positive and significant association between genetic differences (F_{ST}) and geographical distances (km) between pairs of populations.

In the subsequent study (Chapter 3), a similar association between genetic and geographical distances between pairs of populations of *I. scapularis* was found based on analyses of sequence data of the 3' end of the mt 12S rRNA gene and the complete transfer (t) RNA^{Val} gene. As in Chapter 2, significant genetic structuring was detected among tick populations within a geographical region, and 91% of the genetic variation occurred within a tick population. Nonetheless, there were shared haplotypes among populations, which supported the hypothesis of gene flow among them. A total of 62 haplotypes of the mt 12S rRNA + $tRNA^{Val}$ genes were detected among 229 blacklegged ticks, a greater number than that detected when these same

I. scapularis and hundreds more tick individuals were characterized using Domains IV and V of the mt 16S rRNA gene (Chapter 2). Furthermore, it was determined that the observed haplotype richness was lower than expected in both the eastern and western geographical areas. This finding was consistent with that for Domains IV and V of the mt 16S rRNA gene (Chapter 2). Thus, it is possible that there are rare haplotypes that have not been detected in one or more of the populations examined.

The inability to detect rare haplotypes in a population may be a consequence of the sampling protocol used to collect ticks. Significant genetic differences detected among populations of *I. scapularis* may also be due to sampling bias where there may be a greater probability of collecting related individuals at a given site. This potential sampling bias could be reduced by using a standard sampling protocol and collecting ticks on two or more occasions. Also, sampling the same site over several years would provide valuable information on temporal changes in the population genetics of *I. scapularis*. It is important to note that the sample sizes for many of the populations described in this thesis were larger than those used in previous studies. Analyses using larger sample sizes increases the reliability of the estimates of genetic diversity within tick populations. In light of my findings, I recommend that a non-parametric estimator of haplotype richness (e.g., Chao 2) be used in future studies to determine the sample sizes needed to conduct analyses of population genetic structure.

The use of multiple genetic markers to infer population genetic structures and phylogeographical histories is frequently recommended, because of the different levels of sequence variability among markers (Avise 2000, Shao and Barker 2007, Porretta et al. 2013, Araya-Anchetta et al. 2015). Therefore, nuclear genes in *I. scapularis* were subsequently investigated as potential genetic markers. The DNA sequences of the D3 Domain and flanking core regions (= $D3^+$) of the nuclear large subunit (LSU) rRNA gene were determined for 78 *I. scapularis* collected from Minnesota, Manitoba, and Nova Scotia (Chapter 4). The $D3^+$ region was examined as a potential population genetic marker, because of the sequence variation detected among more than 30 *I. scapularis* in the study by McLain et al. (2001). However, in contrast to the findings of McLain et al. (2001), a lack of sequence variation was detected in the $D3^+$ region among the *I. scapularis* (Chapter 4). Moreover, the $D3^+$ sequence of *I. scapularis* (Chapter 4) had little homology with any of the sequences reported by McLain et al. (2001). Later, sequence analyses by Kovalev and Mukhacheva (2012) revealed that the $D3^+$ sequences of

Ixodes persulcatus reported by McLain et al. (2001) were in fact the 28S rDNA sequences of several species of fungi. A similar problem of fungal contamination was reported in the amplification of the $D3^+$ of *Ixodes kingi* (Chapter 4). It is therefore possible that the *I. scapularis* $D3^+$ sequences reported by McLain et al. (2001) represent the amplification of fungal or other contaminants. Furthermore, the $D3^+$ sequences of *I. scapularis* reported in this thesis were homologous to those of other species of ixodid tick, which was not the case for the previously published $D3^+$ sequences of *I. scapularis* (McLain et al. 2001). Nonetheless, my results indicated that the $D3^+$ region of the nuclear LSU rRNA gene does not represent a suitable genetic marker for studying the population genetics or phylogeography of blacklegged ticks, or other species of ixodid tick, due to the lack of intraspecific variation in DNA sequence.

As a consequence, I considered the possibility of conducting population genetic analyses using microsatellite loci. However, few microsatellite loci have been detected in the genome of *I. scapularis* (Fagerberg et al. 2001). Previously, the locus IR27, which has the nucleotide pattern $(AC)_9$, was used by Rosenthal and Spielman (2004) in their study of *I. scapularis*. Since they found nine alleles among 58 ticks corresponding to 15 diploid genotypes, I decided to conduct a preliminary study using this locus. Amplicons from 72 ticks collected from three provinces in Canada (i.e., Saskatchewan, Manitoba, and Ontario) and one state (i.e., Minnesota) in the U.S.A. were electrophoretically compared on Spreadex™ gels (Elchrom Scientific, Switzerland), which can distinguish bands (i.e., alleles) that differ in size by one or more nucleotides. I found that the amplicons of *I. scapularis* from western Canada and the Midwest of the U.S.A. appeared to differ from those in Ontario. However, the characterization of this microsatellite locus was not pursued further or described in this thesis, because cloning and DNA sequencing of these amplicons revealed the presence of at least three alleles at this locus for individual blacklegged ticks. This finding also raised some doubt as to the results obtained by Rosenthal and Spielman (2004), where analyses were conducted on the assumption of a maximum of two alleles for individual ticks at IR27.

In an effort to identify potential alternative targets for investigating the population genetics and phylogeographical history of *I. scapularis*, I characterized the DNA sequences of the complete mt 16S rRNA gene for six blacklegged ticks (Chapter 5). Two of these ticks were previously characterized as haplotype Is-1 based on DNA sequence analyses of Domains IV and V of the mt 16S rRNA gene (Chapter 2). Inclusion of these two individuals was important,

because 45% of the blacklegged ticks in southern Canada were of haplotype Is-1 (Chapter 2). The geographical origins of these *I. scapularis* could not be determined using this genetic marker, because haplotype Is-1 was common in both the Upper Midwest and Northeast of the U.S.A. Each of the six ticks had a unique DNA sequence for the complete mt 16S rRNA gene. This suggested that there were other domains within this gene that could be used to further differentiate among *I. scapularis* individuals, including those of haplotype Is-1. Most (~80%) of the mutational differences in the DNA sequences of the two haplotype Is-1 individuals occurred within Domains I and II (Chapter 5). Hence, the DNA sequences of an ~300 bp region of Domains I and II of the mt 16S rRNA gene was subsequently characterized for 577 *I. scapularis* (Chapter 6). The results of the analyses in this study revealed extensive genetic variability. Intraspecific variation in *I. scapularis* of such magnitude has not been previously reported (Rich et al. 1995, Norris et al. 1996, Qiu et al. 2002, Rosenthal and Spielman 2004, Foley et al. 2008a, Trout et al. 2009, Humphrey et al. 2010, Krakowetz et al. 2011, Mechai et al. 2013, Van Zee et al. 2013). A total of 130 haplotypes were detected, which was two and a half times greater than the number of haplotypes for the same individuals characterized using Domains IV and V of the mt 16S rRNA gene (Chapter 2). Yet, despite this considerable genetic diversity, statistical analyses suggested that the number of haplotypes detected in some geographical areas represented underestimates of haplotype richness. Additional detailed studies are needed to assess the magnitude of this genetic diversity in *I. scapularis*. Nonetheless, the work conducted in this thesis provides the foundation (i.e., the genetic markers) to explore the phylogeography of *I. scapularis* populations in southern Canada.

9.3. Phylogeographical patterns of *I. scapularis*

Previously, Krakowetz et al. (2011) showed that *I. scapularis* from an established population in southeastern Manitoba contained seven haplotypes of Domains IV and V of the 16S rRNA gene, five of which were not detected in four established populations in Ontario or one established population in Nova Scotia. Furthermore, several "new" haplotypes, that is, haplotypes that had not been detected previously in North America, were present within the established populations. Seven of these were only detected in one of three geographical regions (i.e., western, eastern, and Atlantic Canada). Krakowetz et al. (2011) also suggested that there may be different geographical origins for the tick populations in southern Canada. They

hypothesized that the tick populations in the eastern Canadian provinces were derived from colonizing individuals that originated from different geographical regions to those in Manitoba. Although Krakowetz et al. (2011) examined relatively few ticks from some populations, their statistical analyses revealed major differences in the haplotypes present among geographical regions, which further supported their hypothesis. Thus, when commencing my Ph.D. research, I endeavoured to infer the geographical origins of the different populations of this tick species in Canada.

The genetic analyses in Chapter 2 revealed a complete absence of *I. scapularis* individuals of the "southern" clade, the members of which only occur in the southern states of the U.S.A. (Norris et al. 1996, Qiu et al. 2002, Trout et al. 2009, Humphrey et al. 2010). Hence, it is most likely that those *I. scapularis* introduced into southern Canada from the U.S.A. by migratory passerines originated from the Midwest and/or Northeast based on the maximum time that larval and nymphal ticks spend feeding on avian hosts (i.e., 3-5 days) and the maximum distances the birds can fly over this time frame (i.e., 255-425 km) (Ogden et al. 2008e). Therefore, in this thesis, I examined the hypothesis that the distribution of *I. scapularis* in southern Canada is determined by the spring migration routes (i.e., flyways) of passerine birds from the U.S.A. In particular, I tested the hypothesis that *I. scapularis* in western Canada will be more genetically similar (i.e., share more haplotypes) to those in the Midwest, U.S.A. than to those in the Northeast, U.S.A., while *I. scapularis* in eastern Canada will share more haplotypes with blacklegged ticks in populations from the Northeast, U.S.A. than ticks in populations in the Midwest, U.S.A. The data analyses conducted in Chapters 2, 3, and 6 provide support for this hypothesis. Specifically, the Mantel tests (Chapters 2 and 3) revealed a significant positive relationship between genetic differences and geographical distances. This indicates that tick populations in western and eastern Canada were genetically more similar to those in the Midwest and Northeast, U.S.A., respectively, as a function of their greater proximity to these geographical regions. This is also evident upon examination of the information contained within Figs. 2.10, 3.8, 3.13, 6.8, 6.11, and 6.12, which show the number of haplotypes shared among western Canada, eastern Canada, the Midwest, U.S.A., and the Northeast, U.S.A. Characterization of immature *I. scapularis* on migratory passerines at localities along the Central, Mississippi, and Atlantic flyways in both Canada the U.S.A. using the genetic markers defined in this thesis would provide additional support for the hypothesis that blacklegged ticks in different regions of

southern Canada are derived from colonizing individuals that are transported from different geographical regions in the U.S.A. by migratory passerines using different flyways.

The phylogeographic history of *I. scapularis* was inferred in this thesis using sequence data from the three genetic markers: Domains I and II of the mt 16S rRNA gene, Domains IV and V of the mt 16S rRNA gene, and the 3' end of the mt 12S rRNA gene + complete $tRNA^{Val}$ gene. Phylogeographic analyses were also conducted on the concatenated sequence data (Chapters 3 and 6); however, the DNA sequences of the mt 12S rRNA + $tRNA^{Val}$ genes were only determined for a subset (n = 229) of the approximately 580 blacklegged ticks characterized using the different domains of the mt 16S rRNA gene.

The analyses conducted on sequence data for Domains IV and V of the mt 16S rRNA gene (Chapter 2) revealed an association between the presence of some haplotypes and different geographical areas in North America. For example, some haplotypes were detected in both western Canada (Manitoba, Saskatchewan, and Alberta) and the Midwest of the U.S.A., but not in eastern Canada (Ontario, Quebec, and the Atlantic Provinces) or the Northeast of the U.S.A. Similarly, some haplotypes were found in both eastern Canada and the Northeast of the U.S.A (i.e., the "eastern" geographical region), but not in western Canada and the Midwest of the U.S.A. (i.e., the "western" geographical region). These results provided some support for the hypothesis that ticks in different geographical regions of southern Canada have been derived from colonizing individuals that originated in different geographical areas of the U.S.A. Additional support for this hypothesis was garnered from the hierarchical analysis of molecular variance (AMOVA) test, which detected significant differences between geographical regions (i.e., Manitoba, Minnesota, Ontario, and Rhode Island). However, some haplotypes that were detected in both the Midwest and Northeast of the U.S.A. were also detected in southern Canada. For example, haplotype Is-1, which represented 45% of the blacklegged ticks collected from southern Canada, is common in populations in both Minnesota and Rhode Island. Therefore, the geographical origins of ticks that represent these "shared" haplotypes cannot be inferred using Domains IV and V of the mt 16S rRNA gene.

In an effort to provide a greater resolution of the phylogeography of *I. scapularis* in southern Canada, analyses were conducted on sequence data of the mt 12S rRNA + $tRNA^{Val}$ genes (Chapter 3). This objective was achieved because 82 ticks, all of which were previously characterized as haplotype Is-1, belonged to one of 31 haplotypes based on analyses of this

second genetic marker. Furthermore, of these 31 haplotypes, all but three were found either in the "western" or "eastern" geographical region. Nonetheless, the geographical origins of 27% of the *I. scapularis* collected in Canada could not be inferred using this second genetic marker, because some haplotypes were present in both geographical regions. In addition, examination of the minimum spanning network produced from the concatenated sequence data (i.e., for mt 12S rRNA + $tRNA^{Val}$ genes and Domains IV and V of the mt 16S rRNA gene) did not reveal any spatial clustering of the haplotypes based on geographical region. Also, no significant differences in genetic variation between geographical regions were detected by the AMOVA tests. These findings indicating a lack of genetic structure for *I. scapularis* among geographical regions may be a consequence of the small sample sizes used in the study, rather than unsatisfactory phylogeographical signal resulting from sequences of the mt 12S rRNA + $tRNA^{Val}$ genes. This is because the DNA sequences of mt 12S rRNA + $tRNA^{Val}$ genes were determined for a subset of less than half of the *I. scapularis* that were characterized using Domains IV and V of the mt 16S rRNA gene (Chapter 2), and the AMOVA test conducted on the less variable sequence data of Domains IV and V of the mt 16S rRNA gene (Chapter 2) showed significant differences in genetic variation between geographical regions. I suggest that future phylogeography studies targeting the mt 12S rRNA + $tRNA^{Val}$ genes use considerably larger sample sizes to obtain satisfactory phylogeographical signal from this genetic marker.

Additional support for the hypothesis that blacklegged ticks in different regions of southern Canada have been derived from colonizing individuals that originated in different regions of the U.S.A. was provided by analyses of the sequence data obtained for Domains I and II of the mt 16S rRNA gene (Chapter 6). As with the other two genetic markers (Chapters 2 and 3), there were associations between haplotypes and different geographical regions (i.e., "western" versus "eastern"). However, use of this third genetic marker was not sufficient to resolve the geographical origins of all haplotypes in southern Canada, because 23% of the blacklegged ticks were of a haplotype (i.e., Hap_2) that was recorded in six of eleven localities and both the "eastern" and "western" geographical regions. Additionally, there was a lack of spatial clustering of haplotypes in the minimum spanning network, indicating a shallow genealogical structure for *I. scapularis*. When analyses were conducted on the three combined datasets, the minimum spanning network also revealed a shallow genealogical structure for *I. scapularis* (also evidenced by an absence of spatial clustering of the closely related haplotypes

in the network). Thus, the three datasets in Chapter 6 were consistent with the expected life history of a generalist tick species whose dispersal over large geographical areas is facilitated by migratory passerines.

In summary, the collection of studies described in Chapters 2, 3, and 6 provides a better understanding of the genetic structure of *I. scapularis* among different geographical regions in Canada. Major differences in the haplotypes present among geographical regions of southern Canada were detected, which likely correspond to the primary use of a particular migratory flyway by some species of migratory passerines each spring. Major migratory flyways that overlap with the distributional range of *I. scapularis* include the Atlantic flyway, which flows through eastern Canada, including the Atlantic provinces (i.e., New Brunswick, Prince Edward Island, Nova Scotia, and Newfoundland), southwestern Quebec, and southern Ontario (Scott et al. 2012), as well as the Mississippi flyway, which flows through central Canada, including northwestern Ontario and southern Manitoba (Scott et al. 2012). Thus, resident populations of *I. scapularis* in the Atlantic provinces, southwestern Quebec, and Ontario probably originated from neighbouring states along the same flyway in the Northeast, U.S.A. (e.g., Maine, Vermont, and New York and/or even Massachusetts, Connecticut, and Rhode Island). Also, established tick populations in northwestern Ontario and southern Manitoba probably originated from adjacent states along this same flyway in the Midwest, U.S.A. (e.g., Minnesota and Wisconsin). Thus, despite knowledge of major differences in the genetic structure of *I. scapularis* in southern Canada (i.e., that there are differences in the genetic configuration of this tick species among different geographical regions in Canada), the geographical origins of the resident populations remain unknown. Even though the origins of the established populations in Canada remain undetermined, the phylogeographical information obtained from the studies conducted as part of this thesis support the notion that there are still differences in the genetic structure of *I. scapularis* in the different regions of Canada. As blacklegged ticks are important vectors of several pathogens, which are known to cause disease in humans, domestic animals, and wildlife, there is the potential that ticks in the Midwest of the U.S.A. are transporting different strains of bacterial pathogens, as compared to *I. scapularis* in the Northeast. This hypothesis is supported by known and marked differences in the prevalence of two common strains, based on sequences of the 16S rRNA gene, of *A. phagocytophilum* in *I. scapularis* from different parts of the distributional range of *I. scapularis* (Massung et al. 1998, Layfield and Guilfoile 2002, Massung

et al. 2002, Courtney et al. 2003, Massung et al. 2005, Michalski et al. 2006, Steiner et al. 2008, Krakowetz et al. 2014).

9.4. Genetic variation and phylogeographical patterns of *A. phagocytophilum*

At the start of my Ph.D. research, little was known of the prevalence of *A. phagocytophilum* in *I. scapularis* from different regions of southern Canada, or of the diversity of *A. phagocytophilum* strains present in blacklegged ticks. Therefore, one aim of my thesis research was to assess the magnitude of the genetic variability in *A. phagocytophilum* using the 16S rRNA gene and the ankyrin A gene (*ankA*) as genetic markers (Chapter 7 and 8). The molecular assays using the 16S rRNA gene showed that the proportion of blacklegged ticks infected with *A. phagocytophilum* varied significantly among geographical regions and collection years. In addition, two strains of *A. phagocytophilum* were detected: the human pathogenic strain (Ap-ha) (Chen et al. 1994), and the Ap-variant 1 strain, which has not been associated with human infection (Massung et al. 1998). PCR-based assays were developed to distinguish between these strains, which have practical (i.e., diagnostic) implications. The PCR assays conducted on a large sample size of *A. phagocytophilum*-infected ticks revealed that a greater proportion of infected ticks from Manitoba contained the Ap-ha strain than those in more eastern provinces. This finding suggests that the risk of human exposure to the Ap-ha strain of *A. phagocytophilum* may differ among geographical areas in Canada. In addition, the PCR-based assays developed offer a simple and cost-effective solution to monitoring the prevalence of the human pathogenic strain of *A. phagocytophilum* in *I. scapularis* and could potentially be used to determine the strain of *A. phagocytophilum* within human blood.

I also explored the phylogenetic relationships and phylogeographical patterns of *ankA* strains of *A. phagocytophilum* infecting *I. scapularis* (Chapter 8) in relation to other strains of *ankA* reported in prior North American studies (Storey et al. 1998, Pusterla et al. 1999, Caturegli et al. 2000, Massung et al. 2000, Lodes et al. 2001, Von Loewenich et al. 2003b, Dunning Hotopp et al. 2006, Massung et al. 2007, Domingos et al. 2011, Scharf et al. 2011, Katargina et al. 2012, Henniger et al. 2013) and/or deposited on GenBank (see Table 8.1). DNA sequences of this gene have been used to infer phylogenetic relationships (Massung et al. 2000, von Loewenich et al. 2003a, Shukla et al. 2007, Foley et al. 2008a, Scharf et al. 2011) and phylogeographical patterns (Massung et al. 2000, von Loewenich et al. 2003a, Shukla et al.

2007, Foley et al. 2008a) in *A. phagocytophilum*. Analysis of the *ankA* gene of 76 *A. phagocytophilum*-containing samples revealed a total of 13 strains. One of these (i.e., strain CK2) was frequently detected in the western geographical region, while another (i.e., CK7) was common in the central and eastern/Atlantic geographical regions. There was a perfect association between particular *ankA* gene strains and either the Ap-ha or Ap-variant 1 strains of the 16S rRNA gene.

Phylogenetic analyses of *ankA* sequences (DNA and amino acid) revealed three clades, each with strong bootstrap support. Genetically, Clades 1 and 3 (representing the Ap-variant 1 and Ap-ha strains, respectively) were most similar, while Clades 2 and 3 (representing Ap-ha), and Clades 1 and 2 were distinct. In contrast, comparisons of the amino acid residues at the variable positions in the aligned sequences revealed that Clade 1 differed vastly from Clades 2 and 3, possibly due to shifting evolutionary rates and/or physicochemical properties (Studer et al. 2013). Subsequent analysis of the amino acid residues at the variable positions near the N-terminal end of the aligned sequences indicated that Clades 2 and 3, despite differing vastly at the nucleotide level, were biochemically similar (e.g., at some variable positions positively-charged residues were replaced by positively-charged residues). These findings suggested that there may be structural and functional differences in AnkA among the clades.

Phylogeographical analyses of the DNA and amino acid sequences showed that Clades 1 and 2 were comprised of strains that occur in both the western and eastern regions of North America, while Clade 3 was comprised of strains found in only the eastern region. It remains to be determined why *A. phagocytophilum* strains in Clade 3 have not been detected in the western geographical area. One possible explanation is that differences in the distributions of the vertebrate hosts of *A. phagocytophilum* may be influencing the distributions of its different strains (Scharf et al. 2011). Another possible explanation is that the distribution of *A. phagocytophilum* strains is influenced by geographical differences in the seasonal activities of larval and nymphal ticks, such as those previously observed between the Northeast (i.e., bimodal larval activity that is primarily asynchronous with peak nymphal activity) and Midwest (i.e., unimodal larval activity that is synchronous with peak nymphal activity), U.S.A. (Gatewood et al. 2009).

Population genetics analyses were not carried out on the *ankA* data, due to a low prevalence of *A. phagocytophilum* in the *I. scapularis* populations (Chapters 7 and 8). Thus, it

was not possible to determine the particular geographical origins of *A. phagocytophilum* in the tick populations. However, phylogenetic and phylogeographical analyses indicated that there were large genetic gaps among the three clades and that two of these clades are sympatric over a wide geographical area (Chapter 8). The remaining clade (i.e., Clade 3) has only been detected in the eastern geographical area (Chapter 8), suggesting that the populations of *A. phagocytophilum* in western Canada were not likely founded from those in the Northeast region of the U.S.A.

9.5. Comparison of the phylogeographical patterns of *I. scapularis* and *A. phagocytophilum*

I examined whether the phylogeography of *A. phagocytophilum* in North America was congruent with that of *I. scapularis*. The minimum spanning networks for these taxa appear to be incongruous with one another. Specifically, the minimum spanning networks constructed for *I. scapularis*, based on analyses of individual genetic markers and the concatenated datasets, were all characterized by a shallow gene tree and lineages with heterogeneous distributions, as seen in some other animals (Avise 2000). In contrast, the network pattern for *A. phagocytophilum* contained large genetic gaps (i.e., large numbers of mutational changes) between the three clades and contained principal lineages that occurred in sympatry and over a wide geographical area. In particular, the minimum spanning network for this bacterium based on DNA sequences of the *ankA* gene (Chapter 8) showed a phylogeographical pattern typified by a deep gene tree, with most lineages present in both the eastern and western geographical areas (Avise 2000). In order to better resolve the geographical origins of *I. scapularis* in Canada, it may be more useful to examine the phylogeographic patterns of a bacterium present at a high frequency within populations of blacklegged ticks. One possible candidate may be *Rickettsia buchneri*, previously known as the rickettsial endosymbiont of *I. scapularis* (see Kurtti et al. 2015), which occurs at frequencies of 46-100% in populations of blacklegged ticks in the U.S.A. (Moreno et al. 2006, Steiner et al. 2008).

9.6. Synthesis

This thesis contains, to my knowledge, the first collection of studies conducted in Canada targeting multiple genetic markers in both *I. scapularis* and *A. phagocytophilum*. This body of work differs appreciably from related works in the literature, because it contributes to our basic knowledge of the population genetics and phylogeography of *I. scapularis*, as well as the

phylogeography of *A. phagocytophilum*, in Canada. I have shown that several genetic markers are sufficiently variable to partially resolve the phylogeographical patterns among *I. scapularis* and that concatenating the data from these targets increases their resolution and/or the phylogeographical signal offered (Chapters 2, 3, and 6). Furthermore, I have re-examined a previously overlooked genetic marker in *I. scapularis* (i.e., the mt 12S rRNA + $tRNA^{Val}$ genes; Chapter 3), characterized a previously unexplored genetic target in the blacklegged tick (i.e., Domains I and II of the mt 16S rRNA gene; Chapter 6), and identified a new genetic marker with potential for studying the population genetics, phylogenetics, and phylogeographical patterns of the deer tick (i.e., Domain VI of the mt 16S rRNA gene; Chapter 5). I have also discovered that data that is useful for examining the population genetics, phylogenetics, and phylogeographical patterns of a species may also provide insight into functional or phenotypic differences among groups of organisms (e.g., *A. phagocytophilum* belonging to Clades 1, 2, or 3 based on sequences of the *ankA* gene; Chapter 8), opening the door to complementary and/or derivative investigations in the disciplines of microbiology and immunology. Additionally, multiple PCR-based assays have been established to distinguish the human pathogenic strain of *A. phagocytophilum* from the strain that has not been associated with human infection (Chapter 7), which will be useful for monitoring the frequency of the human pathogenic strain in geographical regions where *I. scapularis* has become endemic and/or is in the process of establishing populations. The work reported in this thesis demonstrates the importance of using multiple molecular markers to understand the biogeographical history of a species, as the interpretation of the data may vary depending on the level of variability detected and/or the samples sizes used. Accomplishing these goals and relating my findings to my proposed hypotheses and primary thesis aim should provide some knowledge of the potential risk of exposure for Canadians to the pathogen that causes HGA, as well as, potentially, other pathogens of public health significance (e.g., *B. burgdorferi*, *B. microti*, and the POW virus). Substantial groundwork has been laid from which future population genetic, phylogenetic, and phylogeographical studies of ticks, especially *I. scapularis*, and their associated bacteria, such as *A. phagocytophilum*, may expand upon. The findings in this thesis have implications for future studies on other arthropod vectors and their associated microorganisms whose distributions may also be affected by climate change.

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